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Development and Characterization of Monoclonal Antibodies against Enterohemorragic *Escherichia coli* O157:H7 Lipopolysaccharide.

By

Bozka Malinak Yang ©

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Master of Science in Pharmaceutical Sciences.

Faculty of Pharmacy and Pharmaceutical Sciences

Edmonton, Alberta
Fall, 2003

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Doug Abrams, PhD.

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Date April 4, 2003

Abstract

Twenty years have elapsed after *E. coli* O157 was first recognized as a food-borne pathogen, and this strain constitutes one of the largest threats to the public health, water safety and consumer confidence in the beef industry. Prevalence of *E. coli* O157 contamination indicates that there is a need for a rapid and yet simple technique for diagnosis and treatment.

I hypothesize that a specific anti-LPS monoclonal antibody could efficiently form a homosandwich to detect whole bacteria and shed LPS in contaminated food and water.

Based on a long-term immunization protocol, the glycolipid antigen was used to develop novel P124 hybridomas secreting anti-LPS MAb. In a limited cross-reactivity I demonstrated that the P124 does not bind to three other laboratory strains of *E. coli*. *E. coli* O157:H7 positive samples were identified by epifluorescence microscopy with indirectly labeled P124 anti-LPS O157 monoclonal antibody that can be used for immunofluorescence based water sample testing quantitatively. The anti-LPS O157:H7 MAb was shown to form a homosandwich with the repeated LPS epitopes detected by colorimetric, fluorometric, and chemiluminescence ELISA. Chemiluminescent Femto™ has been shown the most sensitive immunoassay to detect the shed LPS O157 as well as whole bacterium.

Through wisdom is a house builded; and by understanding it is established: and by knowledge shall the chambers be filled with all precious and pleasant riches.

The Book of Proverbs

To

my sister Janka Malinak
and
my husband Yi Yang

I dedicated this thesis

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I owe my warm gratitude to my supervisor, Professor M. R. Suresh, for his guidance, criticism, and encouragement during the course of this study. I am fortunate to be among his students who were introduced to the theory and practice of the biotechnology and immunology.

I would like to express my appreciation to Dr. L. Tang for her valuable suggestions and help in various techniques.

The sincerest thanks to Dr. J. Mercer and Dr. D. Abrams to whom I will always be indebted for assistance in revising my thesis.

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List of Abbreviations

A_{280}	Absorbance at 280 nm
Ab	Antibody
Ag	Antigen
Ag.Ab	Antigen-antibody immune complex
BSA	Bovine serum albumin
^{0}C	Degrees Celsius
CL	Chemiluminescence
CFU	Colony forming unit
CSE	Control standard endotoxin
Da	Dalton
DBSA	Dialyzed bovine serum albumin
DBSA-T	Dialyzed bovine serum albumin-Tween 20
DMEM	Dubelcco's modified eagle medium
E.coli	*Escherichia coli*
EHEC	Enterohemorragic *Escherichia coli*
ELISA	Enzyme-linked immunosorbent assay
EU	Endotoxin units
FBS	Fetal bovine serum
FCA	Freund's complete adjuvant
FITC	Fluorescein isothiocyanate
FL	Fluorescence
GC-MS	Gas Chromatography-Mass Chromatography
GPS	L-glutamine-penicillin-streptomycin solution
HAT	Hypoxanthine, Aminopterin and Thymidine selection medium
HC	Hemorragic colitis
HCl	Hydrochloric acid
HRPO	Horseradish peroxidase
h	Hour, hours

HS	Horse serum
HUS	Hemolytic uremic syndrome
ICSE	Internal control standard endotoxin
Ig	Immunoglobulin
IgG	Immunoglobulin G
IgM	Immunoglobulin M
i. p.	intraperitoneal
i. s.	intrasplenic
kDa	Kilodalton
KDO	3-deoxy-D-manno-octulosonic acid
L	Liter(s)
LAL	*Limulus* amoebocyte lysate test
LPS	Lipopolysaccharides
LMW	Low molecular weight
M	Molar
MAb	Monoclonal antibody
mg	Milligram(s)
μg	Microgram
μL	Microliter
min	Minute(s)
mm	Millimole(s)
MW	Molecular weight
NaCl	Sodium chloride
OD	Optical density
OHCF	Origen hybridoma cloning factor
O-LPS	Smooth lipopolysaccharide
OPI	Oxalacetic acid, sodium pyruvate, bovine insulin
P124	Designated anti-LPS O157:H7 monoclonal antibody
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PBS-BSA-T	BSA in PBST

PBST	0.05-0.1 % Tween 20 in phosphate buffered saline
PEG	Polyethylene glycol
R-LPS	Rough lipopolysaccharide
RT	Room temperature
S. D.	Standard deviation
SDS	Sodium dodecylsulfate
Stx	*Shiga* like toxin(s)
TMB	3,3',5,5'-tetramethyl benzidine
UV	Ultraviolet light
v/v	Volume by volume
w/v	Weight by volume

Introduction

Since first recognized as a human pathogen in 1982, enterohemorragic *Escherichia coli* (EHEC) has become an important dilemma in the health and agricultural sectors of developed countries as well as to the general public (Armstrong *et al.*, 1996). (In recent years, the magnitude and severity of outbreaks have focused the attention of the media to such a level that any *E. coli* O157:H7 infection raises enormous implications for the agricultural and food production industries as well as public's health). Despite the obvious public health significance of *E. coli* O157:H7, much remains to be learned about these bacteria; such as the ability of the organism to survive in feed, water, soil, cattle herds and its adhesion in the intestine. The purpose of this review is to survey scientific approaches in early detection of *E. coli* O157:H7 and potential methods for its effective control.

The second part of the study will be oriented towards practical approaches to develop an *E. coli* O157 specific MAb and demonstrate its utility in detecting whole pathogens and shed antigens.

1. Gram Negative Bacteria *Escherichia coli O157:H7*

1. 1. Metabolism - Growth Requirements

E. coli is a typical member of *Enterobacteriacea,* which are characterized by their ability to grow aerobically and anaerobically. They are able to utilize simple carbon and nitrogen sources for all their metabolic and energy needs. Thus *E. coli* can multiply effectively in a medium containing only glucose, ammonium, and mineral salts (Jensen, Pedersen, 1990).

In many respects, strains of *E. coli* O157 are similar to other *E. coli* strains, but differ in that they do not ferment sorbitol in 24 h (Farmer *et. al.*, 1985) and do not produce β-glucuronidase (Doyle *et al.*, 1984). Because of the inability to ferment sorbitol, *E. coli* O157:H7 grows after overnight incubation in colorless colonies and can be distinguished from most of the remaining intestinal *E. coli* strains that ferment sorbitol and grow as pink colonies on the sorbitol McConkey agar (Karch, 1999). However, sorbitol-positive variants of *E. coli* O157 have emerged. The strains are also β -glucuronidase-positive and produce typical *E. coli* colonies on selective media (Bitzon, *et al.*, 1993). Sorbitol fermenting *E. coli* O157 have been reported from Germany, Hungary, and Czech Republic (Bolton *et al,*, 2000).

The ability of *E. coli* to carry virulence factor genes probably is a selective advantage for growth in a host animal. Martins et al., (1992) demonstrated that 13% of *E. coli* isolated from treated and raw waters carried DNA sequences for one or more virulence factors including the *Shiga*-like toxins, the heat-labile, and heat-stable enterotoxins. *E. coli* strains grow and produce verotoxins over a temperature range of 10 °C to 45 °C. The effect of temperature on recovery and growth may be related to the formulation of the growth medium, which is a critical factor when developing isolation protocols (Bolton *et al.*, 1996). Adherence factors of *E. coli* O157:H7, which had been associated with a water-borne outbreak of hemorrhagic colitis, were shown to be easily obtained from water after many days (Rice *et al.*, 1992). Experimental studies have shown that strains of *E. coli* O157 are more acid-tolerant than other strains and can survive in culture media adjusted to pH 2 for several days (Miller *et al.*, 1994). It has been suggested that enhanced acid tolerance of *E. coli* O157 explains why some strains have caused outbreaks associated with acidic foods such as yogurt, mayonnaise, unpasteurized apple juice, and cider (Buchanan *et al.*, 1996).

1. 2. Classification of *Escherichia coli*

E. coli is one of the most carefully studied Gram-negative organisms. Strains of the various *E. coli* can be identified by presence of specific compounds that are found on the surface of *E. coli* (somatic antigens or O-antigens) and on rigid helical structures that rotate like a propeller (flagella or H-antigens) that extend from the bacterial cell surface. Kaufmann was the first to classify *E. coli* by serological typing of 20 O (somatic) groups in 1944 (Orskow *et al.*, 1972). Since then, the *E. coli* antigen consists of 173 O groups (Lior, 1996). The presence of K antigens was determined when Kauffmann noticed that many live bacteria were not agglutinated by the corresponding O antiserum but became agglutinable when heated. Kauffmann and his collaborators introduced the term K, which stands for the word 'Kapsel' (capsular or envelop antigen). The H flagellar antigens represent specific serological determinants found on flagellin, the protein that constitutes the flagella. Flagella, which are thread-like structures on the outside of bacteria, are motor organs in bacteria or motile organisms (Figure 1). There are at least fifty-six H antigens described for *E. coli.*. Most *E. coli* H antigens are specific and show little or no significant cross-reactivity. A specific combination of O and H antigens defines the serotype of an bacteria. One of these H serotypes, H7, is known as enterohemorragic *E. coli* with a potential as a water and food-borne disease (Hacker, 2000).

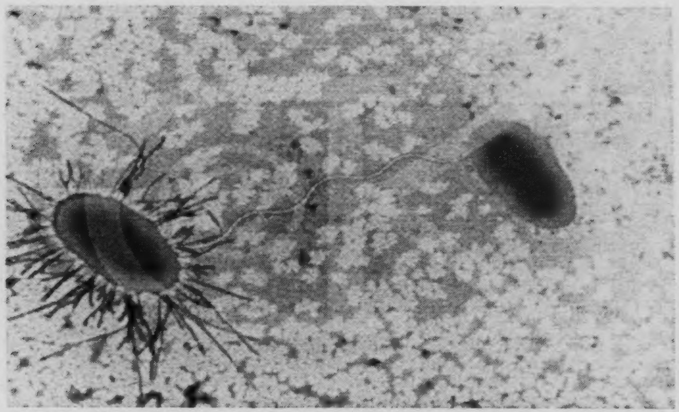

Figure 1: The electron microscopic image of *E. coli* O157:H7
(Taken by T. Oguchi, 2001)

The sequencing data demonstrated that the difference between the pathogenic H7 and H23 flagellins is the result of a single substitution at amino acid (366 Ser -> Thr, Kwang *et al.*, 1998).

The complete sequence of the *E. coli* O157:H7 genome has been revealed. The laboratory *E. coli* K12 strain shares a common 4.1 million-base pair backbone with almost identical gene sequence (Perna *et al.*, 2001). It has been estimated that *E. coli* O157:H7 and *E. coli* K12 shared a common ancestor about 4.5 million years ago.

1. 3. Pathogenesis

There is evidence to suggest that *E. coli* O157:H7 is a relatively new pathogen (Gyles, 2002). Knowledge on the pathogenesis and epidemiology of *E. coli* O157 is mostly based on work with serotype. Like most mucosal pathogens, *E. coli* can be said to follow a requisite strategy of infection: (i) colonization of mucosal site, (ii) evasion of host defenses, (iii) multiplication, and (iv) host damage (Vial *et al.*, 1988). The presence of surface adherence fimbriae is a property of virtually all *E. coli* strains, including nonpathogenic varieties. However, diarrheagenic *E. coli* strains possess specific fimbrial antigens that enhance their intestinal colonizing ability and allows adherence to the small bowel mucous, a site that is not normally colonized (Levine *et al.*, 1984). Once colonization is established, the pathogenic strategies of diarrheagenic *E. coli* strains exhibit remarkable variety. Six classes of diarrheagenic *E. coli* are recognized: enterohemorragic (EHEC), enterotoxigenic (ETEC), enteroinvasive (EIEC), enteroaggregative (EaggEC), enteropathogenic (EPEC) (Figure 2), and diffusely adherent (DAEC).

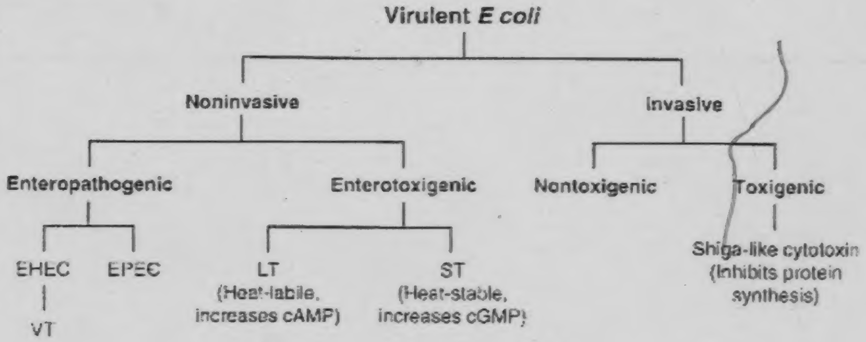
Virulent E coli
Noninvasive
Invasive
Enteropathogenic
Enterotoxigenic
Nontoxigenic
Toxigenic
EHEC
EPEC
VT
LT
(Heat-labile, increases cAMP)
ST
(Heat-stable, increases cGMP)
Shiga-like cytotoxin
(Inhibits protein synthesis)

Figure 2: Virulence mechanisms of *E. coli*

(Adapted from Evans, Med. Microb, Ch.25)

McDaniel and Kaper (1995) have shown that the chromosomal virulence genes of EPEC and EHEC are organized as clustered pathogenic islands of virulence traits. Thus, there appears to be a global regulatory system for regulation of virulence traits that allows *E. coli* O157:H7 to respond to different environmental conditions in different phases of growth. Molecular typing methods revealed that discrepancies between different isolates of *E. coli* O157:H7 are much smaller than genetic differences between other strains of *E. coli.* This would suggest a recent emergence of this organism (Gyles, 2002).

1. 3. 1. *Shiga* Toxins

E.coli strains isolated from diarrhoeal illness produced *Shiga*-like Verocytotoxins. Strains of *E. coli* that produce potent cytotoxins active on Vero cells were first reported in Canada in 1977. Discovery of the Shiga toxins was a result of the necessity to learn more about how gram negative bacterial pathogens cause disease (Konowalchuk *et al.*, 1977), but it was not until 1982 that *E. coli* O157 was first recognized as a human pathogen (Karmali, 1989). The *Shiga* toxins are responsible for the systemic complications of infections and are not required for the initial adherence to epithelial membrane of the gut cell wall by *E. coli* O157:H7 bacteria (Obrig, 1997). Verotoxins consist of two main subunits: subunit A is enzymatically active, and has a catalytic A1 domain and a carboxyl terminal A2 domain, which is responsible for the association of the A subunit with the B subunit. Three saccharide-binding site subunits associate non-covalently to form a doughnut-shaped B subunit pentamer. Hence, the 15 saccharide-binding sites of the B moiety are engaged in binding to the specific toxin receptor on mammalian cells; the glycolipid-globotriaosyl ceramide (Kitov *et al.*, 2000). Once the toxin has gained access to the systemic circulation, the A1 subunit acts as an enzyme halting protein synthesis and causing cell death (Karch, 1999; Thomson *et al.*, 2000).

1. 3. 2. *E. coli* in Diarrhoeal Disease

The recognition of enterohemorragic *E. coli* (EHEC) as a distinct class of pathogenic *E. coli* resulted from two key epidemiological observations. The first was the 1983 report by Riley *et al.*, who investigated two outbreaks of severe bloody diarrhea in 47 individuals in Oregon and Michigan in 1982, with both outbreaks associated with the consumption of undercooked ground beef from the same fast-food hamburger restaurant. The second key observation was by Karmali *et al.* (1983), who reported that once the toxin has gained access to the systemic circulation, microvascular damage might also develop at target organs, thus leading to the clinical picture of hemolytic uremic syndrome (HUS) with fecal cytotoxin and cytotoxin-producing *E. coli* in stool. After the ingestion of contaminated food or water, *E. coli* binds to specific receptors on the colonic mucosal vasculature causing hemorrhagic colitis (Paton *et al.*, 1998). EHEC have two unique characteristics in that they can produce toxins and are capable of colonizing the intestinal tract, where adherence is rapidly followed by invasion of the intestinal epithelial cells (Figure 3). An acute inflammatory response and tissue destruction produces diarrhea with little fluid and much blood (Nataro *et al.*, 1998).

Diarrhea is thought to occur because of toxin action on epithelial cells, and HUS as a result of absorbed toxins acting on renal endothelial cells (Ruggenenti *et al.*, 1998). This is an overly simplistic model where other factors such as cytokines and bacterial lipopolysaccharide are likely to be of an importance (Kerr *et al.*, 2000).

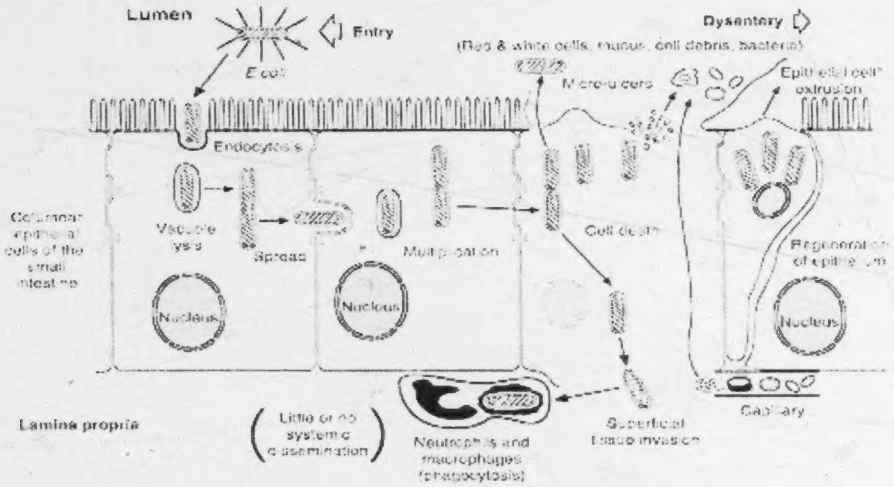
Lumen
Entry
E coli
Dysentery
(Red & white cells, mucus, cell debris, bacteria)
Microulcers
Epithelial cell extrusion
Endocytosis
Columnar epithelial cells of the small intestine
Vacuole lysis
Spread
Multiplication
Cell death
Regeneration of epithelium
Nucleus
Nucleus
Nucleus
Lamina propria
Little or no systemic dissemination
Neutrophils and macrophages (phagocytosis)
Superficial tissue invasion
Capillary

Figure 3: Pathogenic pathway of Enterohemorragic *E. coli* (EHEC)

(Adapted from Evans, Med. Microbiol, Ch. 25)

1. 4. Reservoirs and Source of *E. coli* O157:H7

Several reservoirs and sources of *E. coli* O157:H7 have been identified; for example cattle, deer, and sheep. The bacterium previously thought to grow only in cattle intestine was found in hogs. Gyles reported that 44 herds of hogs out of 100 were found to have *E. coli* O157 strain. However, in the study only three herds were detected for harmful, toxin producing *E. coli* O157:H7 or O157: H-. This is the first time that *E. coli* O157:H7 has been isolated from pig manure in North America (Bennett, 2002). Poultry could be another reservoir for *E. coli* O157:H7 (Ahmed *et al.*, 1995).

Cattle apparently have been a major reservoir of *E. coli* O157:H7 pathogen (Figure 4). The occurrence of *E. coli* O157 H:7 in cattle showed rates of 1.8% (Hanckock *et al.*, 1997), 3.4% (Rice *et al.*, 1997), 7.5% (Van Donkergoed *et al.*, 1999), and 15.7% (Chapman *et al.*, 1997). Studies have found that the highest level of *E. coli* O157:H7 fecal shedding rates occur during summer and early fall, and could be as high as 61% on some farms (Yankee, 2002). *E. coli* O157:H7 levels in calf feces range from 10^2 CFU/g to 10^5 CFU/g. *E. coli* O157:H7 is not pathogenic to calves as inoculation with 10^{10} CFU did not induce significant clinical disease. Oral inoculation of calves with 10^{10} *E. coli* O157:H7 induced prompt and sustained increases in serum antibodies to the O157 antigenic lipopolysaccharide (Chart, 1998). However, *E. coli* O157:H7 does not form attaching lesions and does not colonize mucosal surfaces of gastrointestinal tract. Thus, cattle exposed to *E. coli* O157:H7 are not protected from reinfection (Johnson, 1996). Because dairy farm families are exposed to high levels of bovine verocytotoxigenic from *E. coli* through direct contact with cattle manure and through consumption of unpasteurized milk, they constitute a model of naturally occurring transmission of these organisms from cattle to people (Chapman *et al.*, 1993; Wilson *et al.*, 1998). *E. coli* O157:H7 infection can also spread person-to-person because of the low infectious dose needed. Children under the age of 9 in daycare centers and people over the age of 50 mostly in nursing homes, along with other immunocompromised individuals are at increased risk of secondary attack rate in outbreaks (Slutsker *et al.*, 1997). The largest reported *E. coli* O157 outbreak, which caused illness in more than 8000 people, occurred in Sakai City, Japan in 1996 via contaminated radish sprouts (Buchanan, 1997).

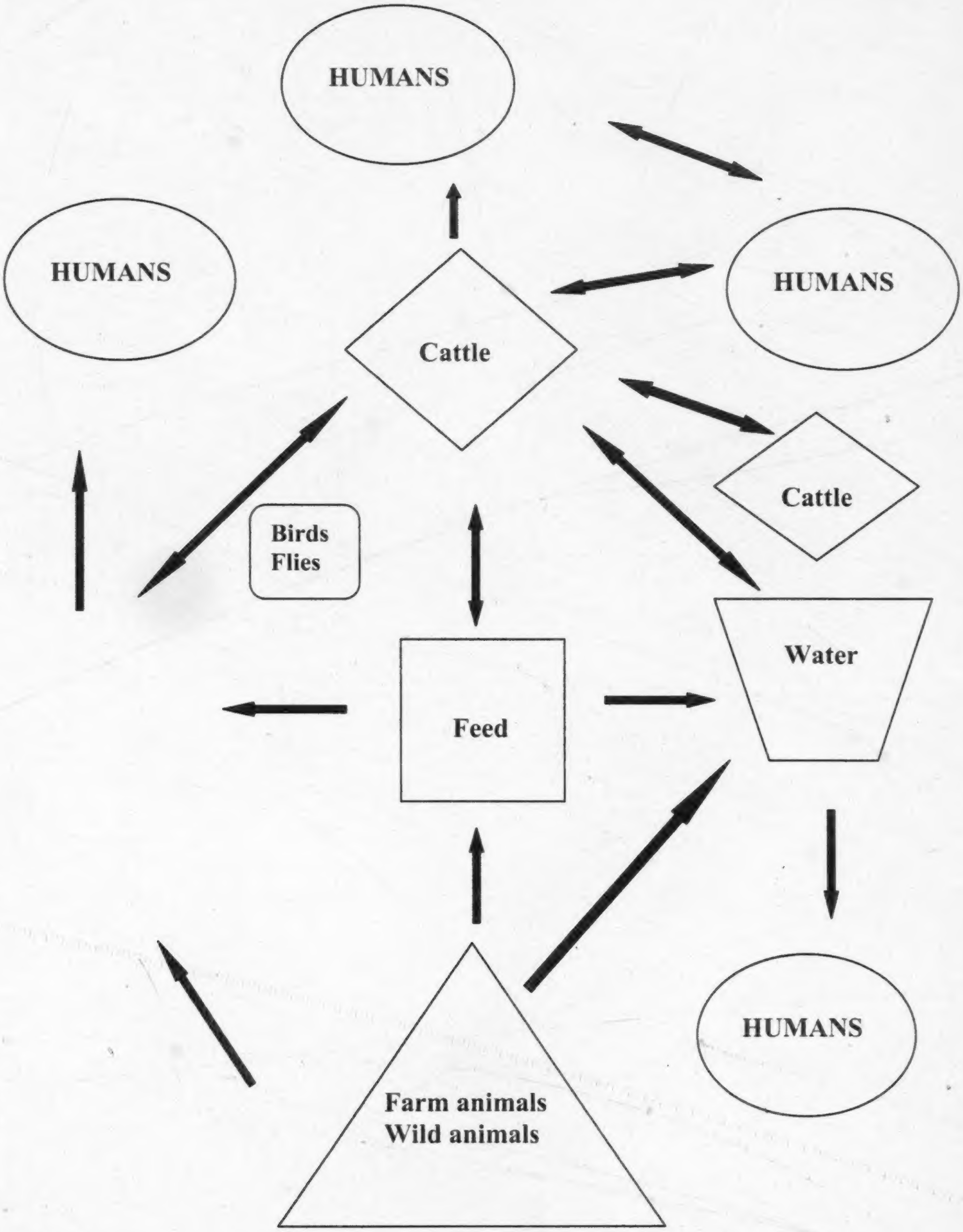
HUMANS
HUMANS
HUMANS
Cattle
Cattle
Birds
Flies
Feed
Water
HUMANS
Farm animals
Wild animals

Figure 4 Modes of Transmission of *E. coli* O157:H7

The first major outbreak in Canada associated with *E. coli* O157:H7 occurred in a nursing home in Ontario in 1985, which resulted in the death of 17 elderly patients (Rodgers, 2000). *E. coli* O157:H7 is perhaps one of the most dangerous enteric pathogens that clinical microbiologists are likely to encounter. Laboratory acquired infections are uncommon, but at least three cases of laboratory-acquired infection with *E. coli* O157:H7 have been reported (Coia, 1998). Laboratories are required by law to undertake local risk assessment to ensure that *E. coli* O157:H7 cultures are handled safely (Bolton *et al.*, 2000).

1. 4. 1. Food-borne Significance of Enterohemorragic *E. coli* O157:H7 Transmission

Outbreaks of infection with *E. coli* O157 have been associated with a variety of food products (Maule, 1999). Contaminated and undercooked ground beef is the principle medium indicated in *E. coli* O157:H7 infections. Occasionally, meat is contaminated with bacteria from the digestive tract during processing (Chart, 1998; Bolton *et al.*, 2000; Elder *et al.*, 2000). Sometimes other food such as dairy products, mayonnaise, and non-fermented apple cider may be contaminated (Feng, 1995).

The evidence from food-borne cases supports the hypothesis of a low infectious dose (Armstrong *et al.*, 1996). Food, that has been indicated as the source of *E. coli* O157 infection, has been reported to contain as few as five organisms per gram (Bolton *et al.*, 1996). Paton (1998) reported the infectious dose of pathogen in contaminated food to be as low as 1 cell per 10 g of meat

1. 4. 2. Water-borne Transmission

Water-borne transmission has been reported from both contaminated drinking water and recreational waters such as children's paddling pools and lakes. In late August 1999, a lake-borne outbreak of diarrhea associated with *E. coli* O157:H7 infection was detected in Clark County, Washington, USA. Seven children were hospitalized; none died. The infection was transmitted and eight secondary cases occurred. This is the largest documented outbreak of *E. coli* O157:H7 infections from recreational water and represents the first time that this strain was isolated from a lake-water sample (Gautom, 2000).

The most serious water-borne outbreak is associated with *E. coli O157:H7* infection in the rural Canadian town of Walkerton, Ontario. In late May 2000, the virulent strain of *E. coli* O157:H7 bacteria entered the town's water system four days after a storm washed manure from a nearby cattle farm into a public well that supplied water for the town. The chlorination system was defective and the contaminated drinking water resulted in death of 11 people and over 1000 cases of serious illness (Rodgers, 2000).

Threats of *E. coli* O157:H7 as a water-borne potential killer escalated the research effort to curtail the risk to humans.

1. 5. Identification and Confirmation of *E. coli* O157:H7

The current recommended procedure for detection consists of testing samples by direct plating and by enrichment culture in modified tryptone soya broth (MTSB) incubated overnight at 37 °C (Wright *et al.*, 1995). An enrichment of the bacteria in tested food products and fecal samples is required since the bacterial excretions as well as the toxin can be very low (Karch, 1999). In addition, traditional culture methods of detection are complemented by various tests utilizing immunologically based methods. Various manufacturers introduce those tests as kits.

1. 5. 1. Human

An important consideration in assessing the burden of *E. coli* O157:H7 disease is its tendency to cause severe hemorrhagic collitis (HC) and hemolytic uremic syndrome (HUS). HC can occur in all age groups, but is more likely to result in hospitalization and death in the elderly. HUS is the most recognizable and serious complication of EHEC infection and provides a useful marker for examining *E. coli* O157:H7 disease trends. Patients with HUS and HC produce serum antibodies to the LPS of *E. coli* O157 (Chart, 1999).

There are three major categories of methods used to diagnose EHEC infections. These are (i) isolation of *E. coli* O157 strains from stool samples; as microbiology laboratories can screen for *E. coli* O157:H7 by inoculating stool specimens onto MacConkey medium containing sorbitol, (ii) detection of *Stx*-producing organisms or fecal *Stx*, (where simple screening methods such as using diagnostic tests that detect *Shiga* toxins are available. Tests for *Stx*-producing organisms are more expensive than culture methods), (iii) detection of elevated antibody levels to O157 LPS in serum (Karch, 1992).

Serological tests can provide evidence of infection. However, due to the long half-lives of the antibodies, they cannot distinguish between active infection and a previous history of infection. The isolation of *E. coli* O157:H7 strains from stool specimens depends upon when stools were cultured. The recovery rate decreases over time, particularly after 6 days of diarrhea. Therefore, by the time HUS has manifested, two of three patients no longer have *E. coli* O157:H7 in their stool (Tarr, 1995). Tests for *Stx*-producing organisms are more expensive than culture methods. There is also instability of the phage carrying the Stx genes (Karch, 1992).

One of the methods involving colony sweeps and immunofluorescence microscopy has been reported as a rapid detection of *E. coli* O157 directly from stool specimens utilizing immunofluorescence stain (Park, 1994).

Several immunologically based methods for detection of the O157 lipopolysaccharide antigen have been developed as alternatives to traditional culture methods. These include immunoassays using dipstick and membrane technologies, direct immunofluorescence on fecal samples, and a commercially available enzyme-linked immunosorbent assay that is used to test fecal suspensions. These have proved to be effective and rapid screening methods for *E. coli* O157 (Park *et al.*, 1994). An immunochromatographic method utilizing gold-labelled antibodies known as GLISA or Premier EHEC Enzyme Immunoassay Singlepath is another rapid test for *E. coli* O157:H7 (Manafi *et al.*, 1999). Antiserum to the O157 LPS and the H7 isolated antigen have been incorporated into various diagnostic kits utilizing ELISA methodology, latex reagents, colloidal gold-labeled antibody, conjugated antibody to fluorescein, peroxidase, or phosphatase (e. g. Meridian Diagnostics, Cincinnati, Ohio; Difco Laboratories, Detroit, Mich; Kirkegaard & Perry Laboratories, Inc., Gaithersburg, Md; Denka Seiken Co., Ltd., Japan; Pro-Lab Diagnostics, Round Rock, Tex.).

Genotypic assays can be used for the identification of a bacterial isolate or for the direct detection of microorganisms in clinical samples. Polymerase chain reaction assays have also been used for screening fecal samples for *E. coli* O157 (Cubbon *et al.*, 1996). Most DNA probes and PCR techniques for *E. coli* O157:H7 are directed toward the detection of genes encoding *Stx.* Recently, the most common plasmid of 60 MDa has been detected by pulsed-field gel electrophoresis (Radu *et al.*, 2001). PCR assays are extremely sensitive. However, in practice, the sensitivity can be influenced by the purity of the target DNA, the presence of inhibitors, and by the technique used to detect PCR products (Karch *et al.*, 1999).

DNA microarray is an emerging technology that utilizes high-density nucleic acid arrays for hybridization. DNA microarray technology was investigated for identification and detection of multiple types of *Shiga* toxin-producing *E. coli* (STEC) and *E. coli* O157:H7 in a single sample. The study demonstrated DNA microarray to be a very powerful tool for studies of multiple genetic events in STEC (Li, 2000).

1. 5. 2. Cattle

E. coli O157 disease can lead into serious health symptoms in humans, whereas *E. coli* O157:H7 infection does not normally cause disease in cattle. A pioneering study on the role of verotoxin in cattle revealed that verotoxin receptor, Gb3, is expressed by the bovine intestinal tract (Hoey, 2002). Verotoxin is also bound to the cell surface in the bovine kidney but not to the vasculature. Data suggest that expression of verotoxin receptor in bovine intestinal epithelium may enhance *E. coli* O157:H7 colonization and thus increase potential distribution to humans.

It has been reported that the autumn application of feedlot pen waste to fields may result in persistence and potential regrowth of bacteria. The studies have demonstrated that pathogenic bacteria has significant persistence in contaminated soil for at least 5 months (Yankee, 2002).

1. 5. 3. Water and Food

The use of immunomagnetic separation has been suggested as a method of reducing total analysis time and improving sensitivity in low dosages of *E. coli* O157:H7 present in the many samples tested (Wright *et al.*, 1994; Yu *et al.*, 1996). Immunomagnetic separation (IMS) utilizes para-magnetic beads coated with antibody to the O157 lipopolysaccharide antigen. The antibody-coated beads are mixed with a portion of the enrichment culture; and *E. coli* O157, if present, become attached to the beads. When the beads are put into a magnetic field, they are attracted to the magnet (Bennett *et al.*, 1996; Tsai *et al.*, 2000). Anti-*E. coli* O157 Dynabeads IMS has been used to isolate bacteria from food and water samples (Yu *et al.*, 1996). The immunomagnetic methods have been modified for the detection and enumeration of *E. coli* O157:H7 bacteria in water samples to detect as few as one living bacteria by using a selective broth for the growth (Shelton *et al.*, 2002).

PCR techniques have also been used to detect as few as 1 CFU of *Stx*-containing *E. coli* O157:H7 in ground beef and other foods after enrichment in broth (Gannon *et al.*, 1992).

1. 6. Current Treatment

1. 6. 1. Humans

The importance of rapid and effective diagnostic protocols and effective treatment can be crucial in cases of *E. coli* O157:H7 infection. There are no known treatments for stopping the infection. There had been some concerns about administer an antibiotic treatment on the risk of HUS being increased or decreased in patients who developed culture-confirmed *E. coli* O157 (Begany, 2000). It has been suggested that antibiotic treatment causes lysis of the *E. coli* O157 with release of additional Shiga toxins (Raghupathy, 1978).

The 1983 Washington State outbreak failed to demonstrate a difference in the antibiotic therapy for HUS symptoms among those who had been given antibiotics compared to those patients without.

Contrary to the Washington study, in the 1996 outbreak of *E. coli* O157 in Sakai City in Japan, Fosfomycin therapy administered within 3 days of the infection reduced the risk of HUS (Buchanan, 1997). However, in evaluation of 71 sporadic cases of *E. coli* O157 infection between 1997 and 1999, an analysis showed that children treated with antibiotics, such as trimethoprim-sulfamethoxazole or a β-lactam, had a higher risk of HUS than children who received no antibiotics (Begany, 2000).

Several studies highlighted the risk of using antimotility agents, in *E. coli* O157 infection (Ryan, 2000). In the 1993 Washington State 'Jack-In-The-Box' outbreak, risk of development of HUS or prolonged bloody diarrhea correlated with administration of antimotility agents; therefore some authors suggest such treatment should be discouraged by physicians (Begany, 2000).

Routine administration of fluids and pain management is appropriate in the case of *E. coli* O157. It has been suggested that *E. coli* O157 conjugates could be used to prepare high-titer IgG anti-LPS globulin for a treatment during an outbreak (Konadu, 1998). Recently, human monoclonal antibodies against *Shiga* toxin 2 were developed as a potential passive immunotherapeutic agent (Mukherjee, 2002).

1. 6. 2. Water

Russian researchers developed a simple electrolyte water treatment technology that may improve the control of *E. coli* O157:H7 in North American cattle feedlots. Preliminary research at the Lethbridge Research Centre utilizing the electrolyzed oxidizing water treatment suggests that *E. coli* O157:H7 pathogen can be totally cleared from water (Alberta Cattlemen's Association, 2002). The Canadian/Swiss Company, Biostel, North America water treatment technology, uses water containing a 0.1% electrolyte solution, which through electrolysis changes the solution pH levels and acquires a unique property to kill pathogenic *E. coli* O157:H7 (Meristem Land and Science, 2002).

Recently, eliminating the pathogenic bacteria in drinking water before they have chance to infect cattle or human has been accomplished by chlorination. There has been active debate regarding the effect of chlorine on cattle and human health. However, proper chlorination was neglected in Walkerton drinking water system with tremendous consequences.

A study showed that *E. coli* O157:H7 could lose O antigenicity after surviving long periods of stress and starvation. Voelker reported that researchers tested *E. coli* O157:H7 in distilled water. They have found that seven of the 19 strains had lost O antigenicity. It has been emphasized that detection methods that do not rely on identifying the *E. coli* O157:H7 O-antigen may be needed to ensure that food and drinking water are safe from the infectious bacteria (Voelker, 2001).

1. 7. *E. coli* O 157 Vaccines

Despite tremendous effort of scientists in many laboratories throughout the world, there are no licensed vaccines against enteric infections caused by bacteria except for cholera and typhoid fever (Robbins, 2001). Vaccine development for *E. coli* O157 has been delayed because there is only indirect evidence of an immune mechanism in humans, and there are no suitable animal models for diseases caused by *E. coli* O157:H7 (Robbins, 2001). The goal of vaccine development would be to curtail animal *E. coli* O157:H7 colonization and limit transmission to humans.

There are three main approaches to the development of a vaccine for *E. coli* O157:H7: one is to use the capsule or the intact bacteria; a second approach is to use a live, attenuated or killed whole-cell strain vaccine, and a third approach is to deliver specific protein antigens, pollysaccharide-protein conjugate vaccines.

1. 7. 1. Humans

A critical level of serum IgG anti-O-specific polysaccharide domain of LPS may give immunity to *E.coli.* The level of natural serum IgG anti-O-specific polysaccharide predicts some resistance to shigellosis. However, polysaccharides are poorly immunogenic (Passwell *et al.*, 2001).

Three vaccines containing detoxified O157 LPS conjugated to *Pseudomonas aeruginosa* recombinant exoprotein A (*r*EPA) as a carrier have undergone Phase 1 evaluation in 87 volunteers age 18- 44 (Konadu *et al.*, 1998). Konadu *et al.* reported a four-fold increase in IgG LPS antibody titers was achieved in 81% of the cases after 1 week and in 100% after 4 weeks. There was a significant decrease in serum IgM anti-LPS at 26 weeks; however, IgG anti-LPS levels were more than 10-fold higher than their pre-injection levels at 26 weeks. Thus the conjugates are promising vaccines, especially for children and the elderly, who are most likely to suffer serious consequences from infection (Soukas, 2000). However it is not known if such antibodies protect against enteric infections after subsequent exposure. The study has been extended for a group of 4- to 7-year-old children. (Passwell, *et al.*, 2001).

Oral immunization with live, attenuated cholera vaccine engineered to express the *Shiga* toxin B subunit induce toxin-neutralizing serum antibody responses with protective immunity to *Shiga*-like toxin I (Acheson *et al.*, 1996).

Shiga toxin toxoid subunit vaccines with plasmid DNA are designed in a way to optimize mucosal immunity. The induction of a mucosal immune response is an important first line defense in preventing disease at the mucosal sites, because *E. coli* O157:H7 enters the host through the specific area on the mucosal surfaces in the gut (Griebel *et al.*, 2002).

Immunological studies of *E. coli* O157:H7 suggest that conjugates composed of the synthetic tetrasaccharide repeating units with a defined heterobifunctional linker conjugated to a protein carrier would be superior to the immunogenicity of conjugates prepared from saccharides extracted from bacteria (Robbins, 2001).

There is a new emerging trend in functional food development and probiotics-live microbial food supplements. The best known are the lactic acid bacteria and bifidobacteria (Macfarlane, 1999). Probiotics have been used to treat or prevent diarrhea. Prebiotics are non-digestible oligosaccharides that can selectively stimulate the growth of probiotic-like bacteria normally present in the gut. Thus, microflora of the large intestine protect against pathogenic bacteria and stimulate development of the immune system (Lacroix, 2002).

Even though some researchers are skeptical of the outcome of probiotics as a vaccine treatment, there is a need for unconventional approaches due to the phenomenon of antibiotics resistance. A large number of microorganisms, including *E. coli* O157:H7, have rapidly developed multidrug resistance to antibiotics that is difficult to control (White, 2002).

In terms of expectations for a human vaccine, the prospect of *E. coli* O157 disease eradication is unrealistic, due to the nature of the reservoir (Gyles, 2002). Unless vaccine-induced immunity was long-lived, it would thus be difficult to make a case for limiting vaccination to children. However, there is indirect evidence that human vaccination against *E. coli* O157 may be effective in preventing illness. A Canadian study of dairy farm families found that infections early in life with non-O157 STEC might be protective against subsequent STEC infection and illness (Canadian Cattlemen's Association, 2002).

1. 7. 2. Cattle

Vaccination may be an effective intervention strategy for reducing carriage of *E. coli* O157:H7 by cattle. The goal of cattle vaccination is reduction of colonization by *E. coli* O157:H7. Thus, effective animal vaccination must necessarily curtail colonization. The Veterinary Infectious Disease Organization (VIDO) in collaboration with the University of British Columbia developed a vaccine that has been in field trials since November 2001. Researchers believe that *E. coli* O157:H7 adheres to the wall of the large intestine by a protein called intimin. *E.coli* first produces its own receptor called translocated intimin receptor (Tir),

which it harpoons into the mammalian cell. The Tir functions as a binding partner for bacterial intimin. The tight binding between Tir and intimin results in int formation of an actin pedestals up to 10 μm in length (Figure 5, Luo, *et al.*, 2000; Goosney, D., Finley, B. 2002). Antibodies are prepared for blocking Tir-intimin binding. Besides the vaccine, the trial examined factors that can affect vaccination efficiency in cattle, such as the use of different management practices and immunization (Babiuk, 2002).

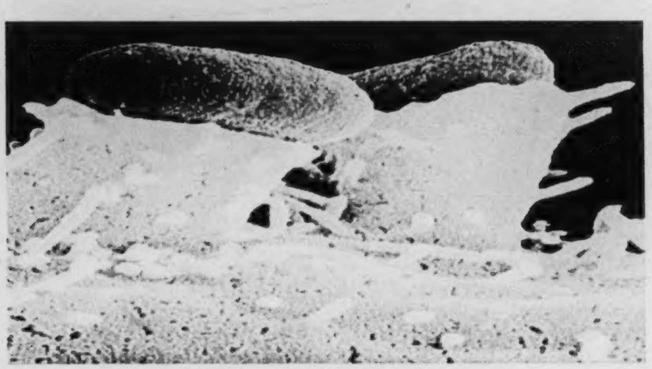

Figure 5: Formation of actin pedestal after intimin adherence into mammalian cells (Adapted from Goosney, D. L., and Finlay, B., 2002)

Colonization of pathogenic bacteria has been shown to be dramatically reduced by mucosal vaccines because they block initial infection. The current vaccination strategies by intramuscular or subcutaneous routes do not induce good immunity at the mucosal surfaces. Another shortcoming of intramuscular injection of vaccine is the economic loss due to tissue damage and consequent carcass trimming at the packing plant. Providing the vaccine as a feed or food supplement has been a significant interest to researchers and beef producers (Babiuk, 2002).

1. 8. Bacteriophage Therapy

While the Western world was focusing on the introduction of new antibiotics to control harmful bacteria, scientists of the Eastern block, especially in the former Soviet Union conducted experiments with naturally occurring viruses possessing the ability to destroy pathogenic bacteria (Osborne, 2000; Pearson, 2002). Bacteriophages are bacterium-specific viruses present in sewage and fecal materials that can invade bacterium. Once inside the cell, the bacteriophage grows, multiples, and eventually bursts out of the pathogenic bacteria (Chanishvili, 2001). Montreal born scientist D' Herrelle promoted the use of bacteriophages for the treatment of infectious diseases almost a century ago (Ackermann, 1982).

Escherichia coli O157:H7 antigen-specific bacteriophages were isolated and tested to evaluate their potency to lyse *E. coli* O157:H7 in the laboratory setting. It has been determined that virulent O157 antigen-specific phages could play a role in biocontrol of *E. coli* O157:H7 (Kudva, 1999; Barrow *et al.*, 1998).

1. 9. Lipopolysaccharide of *Escherichia coli* O157:H7

Lipopolysaccharide (LPS) is a major component of the outer membranes of Gram-negative bacteria (Figure 6). Considerable effort has been made to better characterize the nature of the LPS and determine its precise role in enhancing many biochemical reactions and its interaction with the host (Raetz, 1990). A single *Escherichia coli* contains about 2 million LPS molecules per cell (Raetz, 1990; Ryan, 2001). LPS makes up

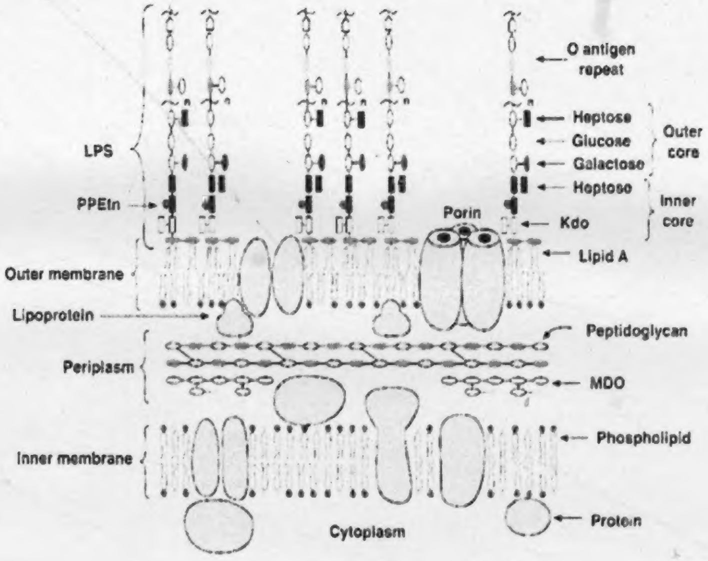
O antigen repeat
Heptose
Glucose
Galactose
Heptose
Outer core
Inner core
Kdo
Lipid A
Porin
LPS
PPEtn
Outer membrane
Lipoprotein
Peptidoglycan
Periplasm
MDO
Phospholipid
Inner membrane
Protein
Cytoplasm

Figure 6: The relationship of LPS endotoxin to the *E. coli* cell surface

Sugar residues are presented as ovals and rectangles, while glycerophospholipids are presented as circles. KDO is 3-deoxy-D-manno-octulosonic acid, MDO is membrane-derived oligosaccharide, and heptose is in the L-glycero-D-manno configuration (Adapted from Raetz, 1996).

a significant fraction of the mass of the outer membrane (~40%), and can extend outward 5-10 nm from the cell surface (Makin *et al.*, 1996).

1. 9. 1. Metabolism of LPS

It is known, that bacteria shed small amounts of LPS in a soluble form, especially by young cultures, into their surroundings while they are actively growing. However, for the most part, the LPS remains associated with the cell wall unit (Todar, 1997). During lysis of the bacterial cells, the LPS molecules are released from the outer membrane into the lysate (Rietchel *et al.*, 1996). The minimal LPS required for the growth of *E.coli* consist of lipid A and two (3-deoxy-D-manno-octulosonic acid) KDO units. O-antigen polypolysaccharides are not required for growth, but supports survival during environmental stresses and plays a major role in the virulence and resilience of the bacteria in the host (Raetz, 1991).

Lipid A is biosynthesized in the cell membrane (Carty *et al.*, 1999). The core sugars are added sequentially to Lipid A. The O-antigen subunits are independently synthesized and are added last. The fully synthesized O-antigen is then attached to the lipid A-core in the cell membrane before insertion into the outer membrane (Fox, 2001).

1. 9. 2. Chemical Structure of LPS

Knowledge of the structure of O-specific polysaccharide of *E. coli* is important in its interactions with antibodies, the LPS binding proteins. The O-antigen could provide protection to the bacteria against damaging reactions with antibody and its complement (Jackman, 2000). LPS is made of three distinct regions: a copolymer of O-specific oligosaccahride repeat units; a core oligosaccharide with a short chain of sugar residues and multiple negatively charged phosphates in the middle; and a hydrophobic lipid A portion at both ends (Jackman, 2000). Lipid A, a unique glucosamine-based phospholipid molecule, is required for bacterial growth and survival. It contains the hydrophobic membrane-anchoring portion of LPS. The length of the O-linked fatty acids located at the 3 and 3' positions differ among bacterial species (Figure 7).

The structure of Lipid A is virtually constant among all *Enterobacteriaceae* and contains two phosphoester groups and hydroxymyristate long chain fatty acids (Odegaard *et al.*, 1997; Wyckoff *et al.*, 1998). The esterified 3-hydroxy fatty acids are frequently used as markers for the classification and identification of gram-negative bacteria (Saraf, 1999). In addition to better characterization of LPS, the lipid A of *E. coli* has been chemically synthesized (Rietchel *et al.*, 1994).

The core antigen consists of a short chain of sugars. Two unusual sugars are usually present: heptose and 3-deoxy-D-manno-octulosonic acid (KDO). KDO is unique and invariably present in LPS; as a result, it has been an indicator in assays for LPS.

The O-antigen is a vital part of the LPS and is the most exposed portion of the smooth LPS, making it highly immunogenic. The hydrophilic domain of O-polysaccharide is much longer than the core polysaccharide. Great variations occur in the composition of sugars in the O-sidechain between strains of Gram-negative bacteria (Perry *et al.*, 1986). The O-chain consists of repeating oligosaccharide subunits made up of 3-5 sugar molecules. The individual chains vary in length and can range up to 40 repeating units (Bundle, Baumann, *et al.*, 1994). The O-specific polysaccharide of *E. coli* O157:H7 LPS is a copolymer composed of tetrasaccharide repeat units (Perry *et al.*, 1986). The absolute configuration of the sugars was determined by GC and GC/MS analyses: D-glucose (Glc), 2-acetamido-2-deoxy-D-galactose (GalNAc), 4-acetamido-4, 6-dideoxy-D-mannose (D-PerNAc), and L-fucose (Fuc) as the copolymer of the repeating tetrasaccharide units in the form of [-4-β-D-Glc-(1-3)-α-D-GalNAc-(1-2)-α-D-PerNAc-(1-3)-α-L-Fuc-(1-]$_n$ (Perry *et al.*, 1986; Nishiuchi *et al.*, 2002).

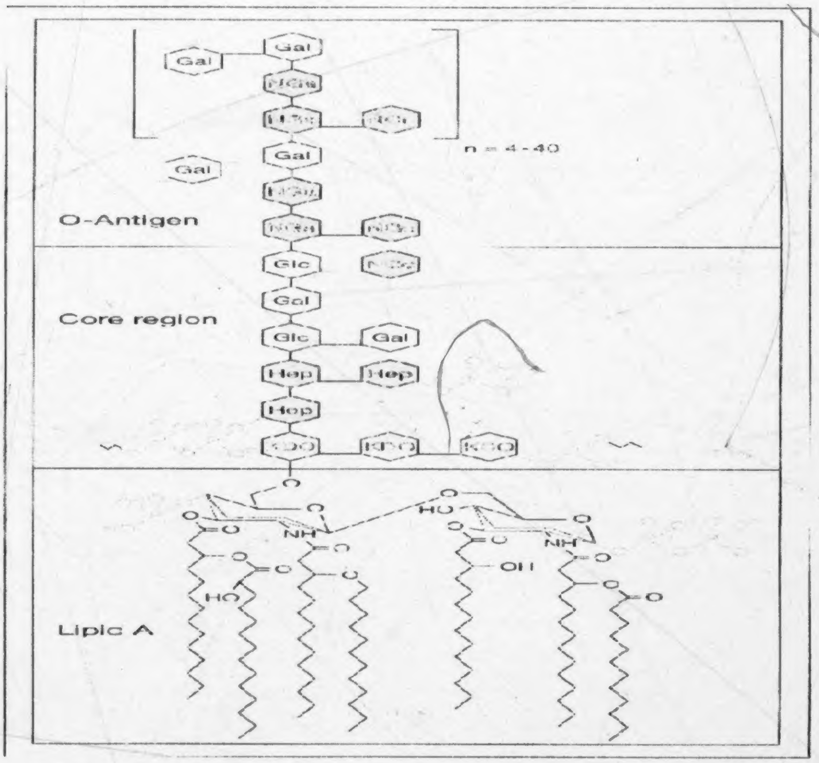
Gal
Gal
n = 4 - 40
Gal
Gal
O-Antigen
Glc
Gal
Core region
Glc
Gal
Hep
Hep
Hep
KDO
KDO
KDO
O
HO
NH
NH
OH
HO
Lipid A

Figure 7: Chemical structure of LPS (Adapted from Todar, 1997)

1. 9. 3. Function of Surface Lipopolysacharides in *Escherichia coli*

The negatively charged LPS provide a number of important functions that are essential for bacterial growth and survival. LPS are connected with itself via divalent non-covalent cation cross bridging and with proteins via both hydrophilic and hydrophobic forces (Hancock, 1991).

The tertiary structure of O- the polysaccharide is important in its interactions with antibodies and LPS-binding protein. The presence of 'smooth' O-antigen is believed to play a major role in enhancing virulence and resilience of the bacteria. Virulence and the property of smoothness are associated with the intact long chain O-polysaccharide (Perna *et al.*, 2001). They are virulent because of the resistance to immune forces of the host. Loss of the O-antigen results in the subsequent loss of virulence, which suggests that this portion is important during the host-bacteria interaction. It is known that phagocytes more readily destroy the core-LPS or fragments of long chain LPS known as 'rough' mutants.

Lipopolysaccharides are complex amphiphilic molecules. Each endotoxin molecule possesses hydrophobic, hydrophilic, and charged regions, which gives LPS its unique features with respect to possible interactions with other molecules. An intact outer membrane is permeable only to hydrofilic molecules. Many hydrophilic molecules greater than the molecular weight of about 700 Daltons and hydrophobic substances such as detergents, bile salts, phospholipases, and other enzymes are excluded or resistant in interaction with the pathogen (Hancock, 1991; Todar, 1997; Hediger, 1994). In addition, the phosphoryl substituents in the heptose region are postulated to be critical to outer membrane stability because their negative charge allows neighboring LPS molecules to be cross-linked by divalent cations (Yethon *et al.*, 1998). The uptake of cationic substances such as antibiotics involves a self-promoted uptake pathway that is initiated by the interactions of these cationic peptides at the divalent cationic cross bridging LPS sites (Rietschel, 1996). The assembly of the heptose portion of the LPS molecule provides potential targets for novel therapeutic compounds (Yethon *et al.*, 1998). LPS may be involved in either colonization, resistance to phagocytosis, or antigenic shifts that determine the course and outcome of an infection (Anup, 1999).

1. 9. 4. Pathogenesis of LPS

LPS is a family of endotoxins (Jones, 2001). Endotoxins are characterized by a net negative charge in solution, high heat stability, and a tendency to form large aggregates in aqueous solution (Dawson et al., 1988). LPS are released during lysis and death of gram-negative organisms (Figure 8). They can induce various pathophysiological reactions in an infected host such as fever, blood clots, and systemic sepsis (Raetz, 1991; Rietchel *et al.*, 1992; Parillo, 1993; Raetz *et al.*, 1996). The lipid A portion is the toxic component recognized by a variety of cells in the body. Important among which are the monocytes, macrophage inflammatory protein, macrophage-derived chemokine, numerous inflammatory cytokines including tumor necrosis factor alpha (TNF), and interleukin (IL)-6, IL-1 alpha, IL-8, growth-regulated oncogene (Rietschel *et al.* 1994; Matsuura *et al.*, 1999; Suzuki *et al.*, 2000). The cascade of those cytokines is dose correlated with the level of endotoxins. It is believed that unregulated overproduction of such proinflamatory mediators rather than LPS itself leads to the physiological changes observed during a sepsis systemic inflammatory response (Jones, 2001).

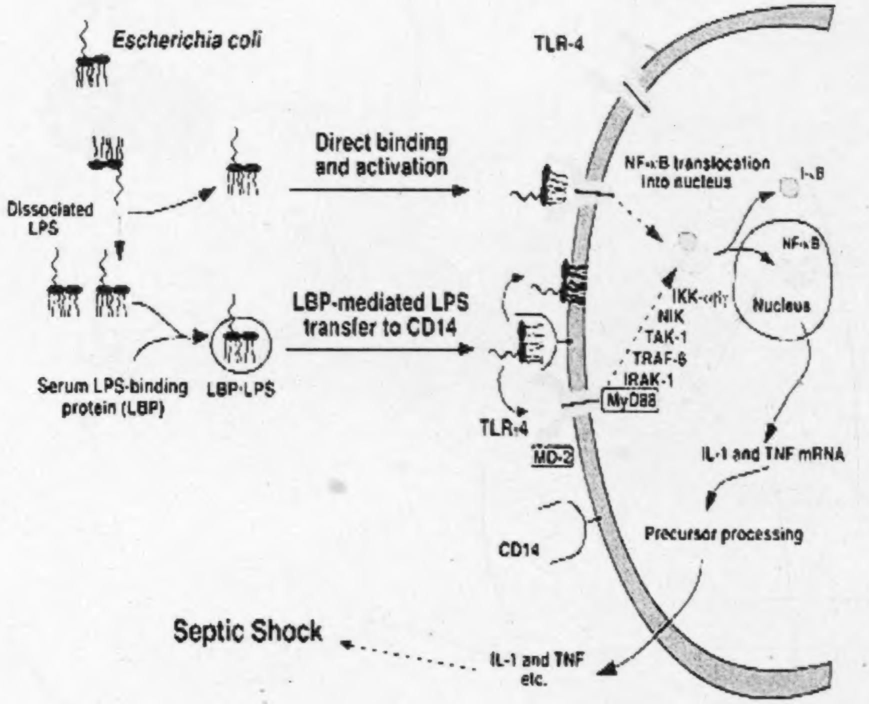
Escherichia coli
TLR-4
Direct binding and activation
NF-κB translocation into nucleus
I-κB
Dissociated LPS
NF-κB
LBP-mediated LPS transfer to CD14
IKK-α/β/γ
NIK
TAK-1
TRAF-6
IRAK-1
Nucleus
MyD88
Serum LPS-binding protein (LBP)
LBP·LPS
TLR-4
MD-2
IL-1 and TNF mRNA
Precursor processing
CD14
Septic Shock
IL-1 and TNF etc.

Figure 8: Pathophysiological reactions to LPS

(Adapted from Raetz, 1996)

1. 10. Hybridomas and Monoclonal Antibodies

1. 10. 1. Antibody Structure and Immunologic Diversity

Antibodies belong to the family of naturally occurring glycoproteins in serum called immunoglobulins (Smith, 1998). Immunoglobulins are secreted from activated B-cells in response to invading toxic agents or foreign substances; and specifically bind to those agents. The typical immune response is extremely heterogeneous. Clones produce antibodies of a great range of structures both in their binding regions as well as in their effector regions. It is possible to distinguish two types of domains: variable (V) domains and constant (C) domains. Molecular integrity is maintained by specific covalent bonding mediated through interchain disulfide bridging and by strong noncovalent interactions between the two heavy-chains and between each of heavy-chain/light-chain pairs. All humans and mouse immunoglobulins (Ig) are divided into five major classes, IgG, IgM, IgA, IgE, and IgD. They are large, ranging from 150 000 to 950 000 Daltons depending on their class.

IgG is the simplest antibody among all five classes. IgG antibodies are usually the most important serum immunoglobulin in mammals; accounting for more than 80% of total serum immunoglobulins produced on repeated exposures to an antigen. Each IgG molecule consists of two identical short peptide chains called light chains and two long polypeptide chains called heavy chains (Figure 9).

IgM occurs both on the surface of B-cells as a monomer and free in serum as a pentamer (Figure 10). It is the first class of antibodies to appear after initial exposure of an antigen to an individual. Its 10 identical antigen-binding sites allow it to bind to antigens avidly and to efficiently activate complements so that the antigen may be quickly destroyed.

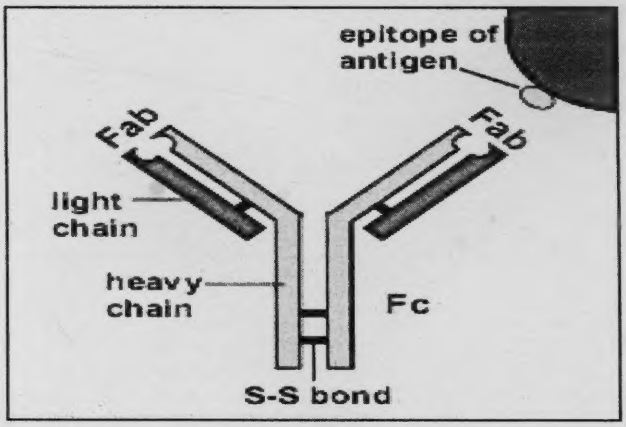
epitope of antigen
Fab
Fab
light chain
heavy chain
Fc
S-S bond

Figure 9: Immunoglobulin G structure

(Redrawn from diagram by Bentzen, 1998)

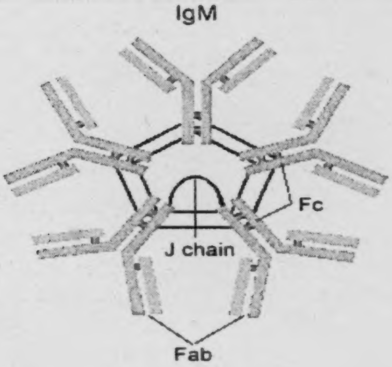
IgM
Fc
J chain
Fab

Figure 10: Immunoglobulin M structure

(Redrawn from diagram by Bentzen, 1998)

A broad spectrum of antibody molecules differ in both the amino acid sequence of their variable region and in the affinity with which they recognize the antigen. The interaction of an antibody with an antigen forms the basis of all immunochemical techniques. The N-terminal 110 amino acid residues of both the heavy and light chains of the immunoglobulin molecules are variable in sequence and interact to form the antigen-binding site. About 30 amino acids are potentially available as antigen contact residues, but X-ray crystalographic studies have shown that a maximum of only 17 amino acids are directly involved in binding contact (Selby, 1999). Nevertheless, this variability gives rise to a vast array of different antibodies binding to different molecules. These reactions maintain high specificity and no chemical change is introduced in the antigen.

1. 10. 2. Monoclonal Antibody Production

In 1975 Kohler and Milstein reported the first successful cell line to secrete antibodies which had a specificity for a pre-determined antigen. It is known that antigen-stimulated spleen cells derived from a mouse fused with myeloma cells in polyethylene glycol results in hybrid cells that can be cloned in specialized medium, which only allows the growth of the hybrid cell clones. Fusion of cells between an antibody-producing spleen cell and an immortal myeloma cell can be cloned and continuously cultivated. This resulted in hybridomas secreting monoclonal antibody with practical applications (Ishikawa, 1998; Kohler and Milstein, 1976). For the first time naturally occurring antibodies were treated as chemicals instead of a variable biological serum product (Borrebaeck, 2000). With the use of fused hybridoma cells it was possible to provide an unlimited source of highly specific homogenous antibodies.

Although Milstein and Kohler had predicted the biomedical applications of monoclonals in their historic 1975 paper, the practical utility of monoclonal antibodies as diagnostic and therapeutic agents were realized in the next two decades. Diagnostic applications were clearly the first series of applications that became popular. The introduction of antibody engineering resulted in the production of antibodies with novel modifications including bispecific antibodies (Winter, 1991). In other cases, the monoclonal antibody is conjugated to another molecule such as a fluorescent molecule to aid in the imaging of the target or labelled with a radioactive atom, such as iodine-131 to aid in the killing of the target.

1. 10. 2. 1. Cell Fusion

The success of the delicated process of cell fusion depends on several factors such as healthy spleen cells, the quality of myeloma cells, the amount of polyethylene glycol (PEG), and the length of incubation time of PEG with the cells. In addition to those variables is the use of Sendai virus or PEG to make possible the fusion of adjacent plasma membranes, resulting only in a small percentage of the fused cells. Some of these fused cells are homofusions between two myeloma cells or two B cells rather than the desired heterohybridoma.

The myeloma cell lines selected for hybridoma fusion share one common feature – they can be killed in a selective tissue culture medium. Myeloma cell lines with thymidine kinase deficiency will be killed in bromodeoxyuridine selection medium (Wong *et al.*, 1987). The most commonly used selection system is the hypoxanthine, aminopterin, and thymidine (HAT) system with hypoxanthine guanine phosphoribosyl transferase deficiency generated in meloma cells (Suresh *et al.*, 1986).

HAT selection depends on the fact that mammalian cells can synthesize nucleotides by two different pathways, the *de novo* or the salvage pathways. In the *de novo* pathway, cells can use a methyl or a formyl group transferred from an activated form of tetrahydrofolate to synthesize nucleotides. When the *de novo* pathway is blocked by aminopterin, cells will use the salvage pathway by converting preformed purines and pyridines directly into DNA and RNA. The hypoxantine-guanine-phosphoribosyltransferase (HGPRT) enables cells to synthesize purines using extracellular source of hypoxanthine as a precursor.

It is known that SP 2/0 is a myeloma cell line that has lost the ability to synthesize HGPRT (Figure 11). The absence of HGPRT is not a problem for normal cells because they have an alternative pathway that they can be used to synthesize purines. However, when cells are exposed to aminopterin (a folic acid analog), they are unable to use the salvage pathway and are now fully dependent on HGPRT from the normal spleen cell for survival. Therefore unfused myeloma cells or homofusions between myeloma cells will not survive in HAT medium.

Additional important characteristic of the SP 2/0 is that they have lost the ability to synthesize any antibody molecules. The hybridoma cells produced by successful fusions are able to grow indefinitely because of the spleen B cells that have the wild type HGPRT enzyme and the myeloma partner is immortal. Further the unfused or homofused spleen cells do not need to be separated because they are only capable of limited growth in vitro (Figure 12).

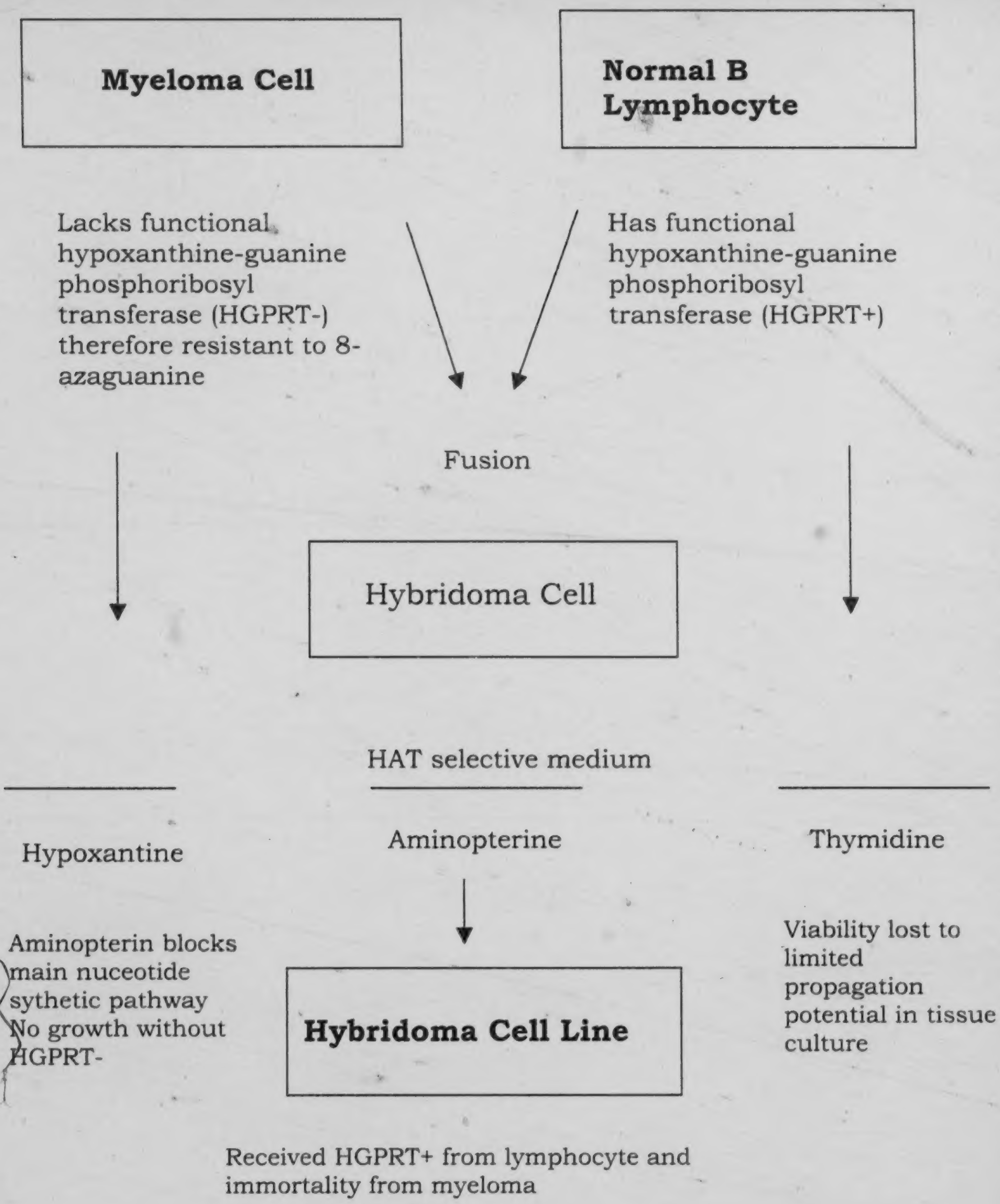
Myeloma Cell
Normal B Lymphocyte
Lacks functional hypoxanthine-guanine phosphoribosyl transferase (HGPRT-) therefore resistant to 8-azaguanine
Has functional hypoxanthine-guanine phosphoribosyl transferase (HGPRT+)
Fusion
Hybridoma Cell
HAT selective medium
Hypoxantine
Aminopterine
Thymidine
Aminopterin blocks main nuceotide sythetic pathway No growth without HGPRT-
Hybridoma Cell Line
Viability lost to limited propagation potential in tissue culture
Received HGPRT+ from lymphocyte and immortality from myeloma

Figure 11 Cell fusion flow diagram

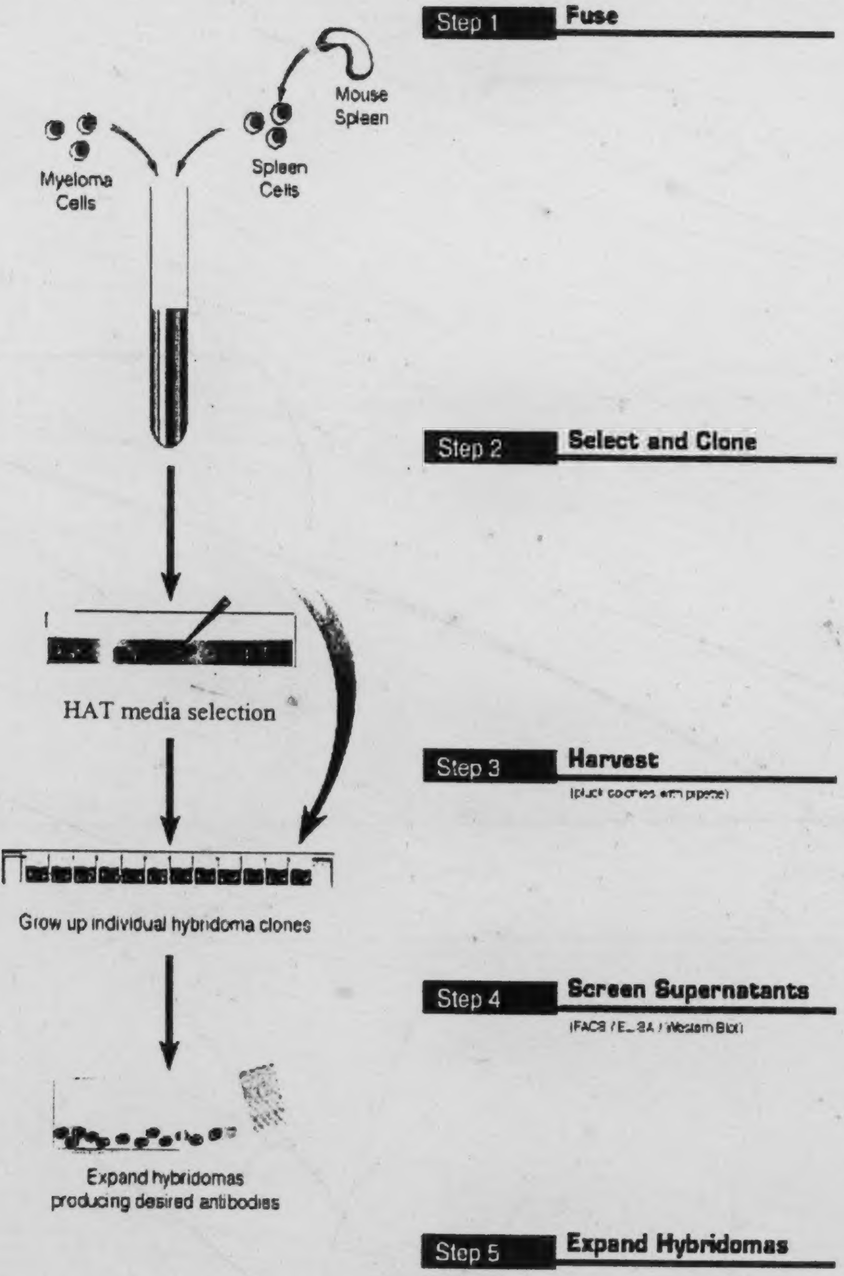
Step 1
Fuse
Mouse Spleen
Myeloma Cells
Spleen Cells
Step 2
Select and Clone
HAT media selection
Step 3
Harvest
(pluck colonies with pipette)
Grow up individual hybridoma clones
Step 4
Screen Supernatants
(FACS / ELISA / Western Blot)
Expand hybridomas producing desired antibodies
Step 5
Expand Hybridomas

Figure 12 Monoclonal antibody production

(Adapted from www. AntibodyResourcePage)

1. 11. Development of Ultrasensitive Immunoassays

The immunoassay technique has proved to be a prominent method in biotechnology and clinical research, contributing to the understanding of diseases and leading to the development of useful diagnostic tests. It is possible to develop an immunoassay specific for almost any molecule as long as a specific antibody to this molecule is available. Since Yalow and Berson first elucidated the principles of immunoassay in 1959 there has been continuous growth in immunoassay development.

1. 11. 1. Specificity

Specificity is one of the most important requirements of immunoassays. Evaluation of specificity is a very important step in the optimization of every new immunoassay. In determining the overall specificity of an assay, a major factor is the cross-reactivity of the antibody. Sources of cross-reactivity are due to the ability of other molecules with structurally similar or identical epitopes to bind to the antibody. One approach to reducing cross-reaction is by careful selection of the immunogen to develop more specific antibodies. Assessment of antibody cross-reactivity is determined by incubation of a fixed antibody concentration with varying concentrations of an analyte, their relative antibody-binding affinities with regard to many binding sites in the case of the cells or microorganisms (Selby, 1999). It is usual practice to employ a monoclonal antibody selected by epitope mapping to react only with predetermined sites on the antigen molecule.

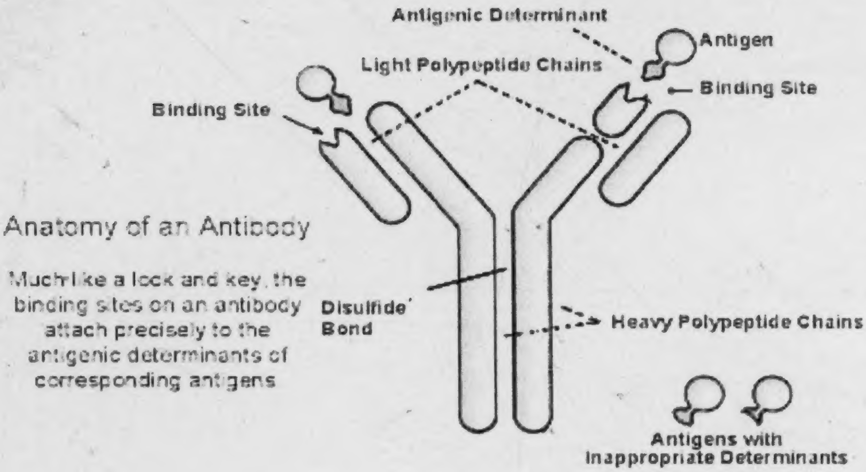
Antigenic Determinant
Antigen
Light Polypeptide Chains
Binding Site
Binding Site
Anatomy of an Antibody
Much like a lock and key, the binding sites on an antibody attach precisely to the antigenic determinants of corresponding antigens
Disulfide Bond
Heavy Polypeptide Chains
Antigens with Inappropriate Determinants

Figure 13: Antibody-antigen interactions

(Adapted from diagram by Honegger, A., Department of Biochem, Zurich University).

1. 11. 2. Sensitivity

One of the fundamental characteristics of any analytical method is the smallest concentration that can be accurately distinguished. The aim of any analytical method is the achievement of high sensitivity. The continuous increase of sensitivity in immunoassay remains one of the most important parameter in assay binding technique. Throughout the 1950s and early 1960s there was contraversy regarding the definition of sensitivity. Even as late as 1998, researchers still had differing definitions of sensitivity (Selby, 1999; Ekins, 1998).

Yallow first defined the slope of the calibration plot of stimulus reading versus stimulus. The sensitivity is associated with the change in the response of enzyme activity rate measurements for a small change of the concentration of stimulus. To determine the sensitivity of analyte detection, the signals generated for two or more amounts of analyte that differ by small concentration are recorded. The slope method is formulated to be applicable to the widest possible range of situations. For example, in initial calibration-rate, determination of enzyme activity measurements is influenced by variables such as instrumental noise, temperature, and pH. However, Ekins concluded that the slope alone is not an index of assay sensitivity. Instead, assay sensitivity is defined as the imprecision of measurement of an analyte concentration in a control sample with no analyte (Ekins, 1998). This in effect reflects the degree of noise in the assay. Detectable analyte concentration or detection limit or sensitivity of an immunoassay is defined as the analyte concentration that gives a response which has a statistically significant difference from the response of the zero analyte sample. Ekins states that a simple and straightforward method to define assay sensitivity is to use the precision of the zero dose estimates. Nonetheless, there is no universal agreement regarding how the precision of the zero dose estimates should be defined.

The formal definition of analytical sensitivity is formulated according to Ekins's concept as the amount of analyte required to produce a change that can be distinguished from background noise (zero dose of analyte). This concentration is properly termed the assay's detection limit known also as sensitivity. Sensitivity is established by assaying replicates of a sample that is known to have no analyte. Then the measured signals from these replicates are used to calculate a mean and standard deviation. The analytical sensitivity is determined as the mean signal obtained from the zero sample plus 2 standard deviation for immunometric assays (Ishikawa, 1998).

The fundamental difference between the two concepts is whether or not detection limits dependence on the error in the measurement.

Ekins has been the most vocal advocate of the meaning of sensitivity (Ishikawa, 1998; Ekins, 1998).

1. 11. 2. 1. Ultrasensitive Immunoassay

Further extensive experimental work resulted in the introduction of a second generation of non-competitive immunoassays with ultrasensitive potential detection of analyte. The description "ultrasensitive assay" refers to a microanalytical technique classified as a second-generation non-competitive method. The terms "ultrasensitive" and "second generation" have not been defined, but often appear to be used in practice to describe marginal improvements in sensitivity (Ekins, 1998).

1. 12. Two-site Immunometric Assay

These assays are applied to the determination of macromolecular antigens, where simultaneous binding of two antibodies to the antigen is allowed without steric obstructions. All immunoassays depend on the measurement of fractional occupancy of the sensor antibody (Ekins, 1994). The solid phase antibody is regarded as the sensor antibody and the second antibody is either conjugated to an enzyme as a reporter antibody or coupled though an adapter with a third antibody-enzyme complex by utilizing variable combinations of biotinylated antibodies and streptavidin-enzyme complex (Diamandis, 1991). The sensor antibody-binding sites are occupied by the antigen to a fractional extend that depends on the equilibrium constant of the binding reaction and the final unbound antigen concentration in the mixture. The Mass action law can describe the reaction between antibody and analyte:

$$[AbAg]/[fAb] = K\,[fAg]$$

where [AbAg] represents concentration of bound antibody, [fAb] is concentration of free antibody, [fAg] is concentration of free antigen, and K is equilibrium constant. If the total antibody concentration [Ab] approximates 0.01/K - 0.05/K, then free [fAg] and total [Ag] concentrations do not differ significantly and fractional occupancy depends only on the reaction between sensor antibody and analyte (Ekins, 1994). The resulting occupancy of antibody binding sites is an indicator of the concentration of antibodies in the medium and is independent of the total antibody concentration present in the sample. Thus, all immunoassays depend on measurements of the fractional occupancy of the sensor binding sites and can be measured either directly or indirectly. The signal of related antigen of which occupied sites are directly measured using relatively high sensor-antibody concentrations can be described as non-competitive.

In a single-site labeled antibody assay, the labeled antibody itself constitutes the sensor antibody, which may be separated into occupied and unoccupied fractions. After the separation of bound and free-labelled antibody the signal emitted by labeled antibody bound to analyte is measured directly.

1. 13. Interference in Immunoassay

When evaluating a new immunoassay for validity several criteria are important. The immunoassay must be reproducible, it must be able to detect analyte concentrations that corelate with clinical disease and the interpretation of data must clearly relate to the clinical reality. Studies have shown that interference, which can be analyte-dependent or analyte-independent with false positive or false negative results, limits the sensitivity of immunoassays (Kricka, 1998; Price, 1998).

1. 13. 1. Blocking

In an ELISA, specific ligands (antigens or antibodies) are immobilized on polystyrene surfaces by non-specific binding. Saturating the polystyrene with inert molecules can minimize remaining binding of adsorptive surface and lower background. Blocking proteins have no active part in the immunochemical reactions of the assay. Many factors can influence non-specific binding, including various protein-protein interactions that are specific to a particular ELISA system. Steinitz (2000) reported, that the presence of blockers and detergent could eliminate interference from plasma protein. It has been reported that the use of binding blockers may change the binding characteristics of antibodies, especially monoclonal antibodies. Gosling (1990), along with other researchers, abandoned using protein such as bovine serum albumin to block unoccupied sites on solid-phase surface coated with antibody in the immunoassay analysis. Recently, the positive effect of bovine serum albumin in addition with Tween 20 has been reported

(Drexler, 1986; Wu, 2000). The assessment of adequate concentration of detergents is conducted due to denaturation of protein and desorption of antibodies from solid phase with excessive concentrations of Tween 20 or Triton X-100.

1. 13. 2. Immobilization of Substance on the Surface of Solid Phase.

Proper utilization of hydrophobic, hydrophilic and covalent bonding forces of the polystyrene-irradiated microtiter plate will be beneficial during evaluation of the design assay with the final result of maximum protein-plastic hydrophobic bonding.

Researchers have spent considerable time investigating the direct and efficient immobilization of the substance onto the polystyrene surface. The complexity of the task can be illustrated in the case of the immobilization of viruses and bacteria as solid-phase reactants. Polysaccharides and heavily glycosylated proteins (e.g. viruses and bacteria) often have low affinity for polystyrene and often require alternative methods for immobilization. Several successful surface modifying agents have been used as a pretreatment of the polystyrene surface (Weiping, 2000). In addition to treatment with Triton X-100, it has been demonstrated that coating can be improved by pretreating the microwells with poly-L-lysine (Wu, 2000). Preparing highly stable antibody surfaces by immobilizing antibodies on polystyrene surfaces was evaluated in correlation to the level of roughness. It has been concluded that the topography of the surface along with characteristics such as hydrophobicity and surface charge are important for antibody adsorption and can improve the sensitivity of the immunoassay (Weiping, 2000).

Some of the key players in establishing optimal immunoassay conditions are the pH of the buffer and the ionic strength of reagents used in the sample, especially in the case of monoclonal antibodies. Proteins are usually incorporated in the buffer along with a detergent. It has been reported that coating microtiter wells with 100μ L of analyte in the presence of 0.01% Triton X-100 significantly enhanced the immobilization of the antigen to the microtiter wells (Wu, 2000).

1. 13. 3. Hook Effect

Generally, the binding of an antigen to the plastic carrier will be through hydrophobic regions on the molecule. A large multivalent antigen complex will have many more attachment sites per molecule entity and leads to increased strength of the antigen-carrier interactions. However, this increased binding does not always lead to higher readings in the ELISA. Rotmans observed that changes in the shape of the antigen molecule due to binding to the plate, decreases the immunoreactivity of the antigen (Rotmans, *et al.*, 1984). The decrease in the ELISA reading could be further influenced at very high concentrations of analyte. When the antigen concentration is extremely elevated, it may saturate both the solid phase-bound primary antibody and the detecting antibody. This prevents the formation of detectable sensor antibody/analyte/reporter antiboby complexes, resulting in a decrease in the assay value. For most sandwich types of assays, whenever there is an insufficient amount of antibody coated on the solid phase, the dose-response curve is likely to result in a bell-shaped curve. The curve rises initially and then changes direction at certain points of high analyte concentration. Thus, instead of continuously rising, it falls again. Because the curve is roughly in the shape of a hook, this phenomenon is called the “hook effect”. The effect was first noticed in two-site immunometeric assay by Miles in 1974.

The hook effect is most commonly found in the single-step immunometeric assay; a popular format, chosen for its specificity and speed. In a single-step reagent-excess assays, the captured antibody and analyte are mixed and a signal antibody is added. The huge excess of analyte is thought to saturate both specific and less specific binding sites on both antibodies, thereby preventing formation of the sandwich. The incidence of a decrease in ELISA readings can be partially eliminated by a sufficient washing prior to addition of the second antibody, and avoiding simultaneous saturation of both antibodies.

Despite attempts to eliminate or reduce the hook effect by careful assay design, the only reliable method of routinely eliminating the hook effect is by the analyzing of all samples at several dilutions to find a suitable dilution.

1. 14. Multivalency in Immunoassay

The interaction of an antibody with an antigen forms the basis of all immunochemical techniques. Immunoassays are a form of macromolecular binding reaction. Reversible binding of antibody to antigen is dependent on a combination of many types of noncovalent interactions, including formation of hydrogen bonds between adjacent amino, hydroxyl, and carboxyl groups at distances 1.5-5 Angstrom. Weaker van der Waals forces could also be involved where electrostatic interactions are formed due to electron spin effects.

Unique to the antigen and antibody interaction are the multiple binding sites often possessed by both molecules. IgG has two binding sites. Many antigen molecules have multiple antigenic sites. Therefore, extensive cross-linking of antigen and antibody may occur when soluble antigen and its antibody are mixed in solution.

Multivalent interactions can greatly stabilize the immune complex (Ag.Ab), rendering the reactions practically irreversible.

$$Ag + Ab \longleftrightarrow Ag.Ab$$

It is known that multivalent interactions allow low-affinity antibodies to bind tightly. An antibody bound to an antigen immobilized on a solid support is one example. In this case, the high concentration of antigen coated on the solid phase results in the increased strength to the interaction. All immunoassays are based on these unique properties of antibody and antigen molecules.

1. 14. 1. Multivalent Carbohydrate-Antibody Bindings

Cell surface carbohydrate epitopes are markers of bacteria and therefore represent important targets for diagnostic and therapeutic antibodies (Figure 14). Carbohydrate-protein interactions mediate a wide range of biological processes that are vital to the normal physiological functions and developments that lead to the onset of disease by cell surface adhesion of bacteria and bacterial toxins (Luo and Finley, 2000).

In nature, the interactions of carbohydrates with other carbohydrates and proteins are of fundamental importance to many biological processes, such as immune defense. Protein-carbohydrate interactions are relatively weak. In nature, the problem of weak affinity is solved by displaying multiple copies of both carbohydrate ligands and their protein receptors on the surface of interacting cells. The weak interactions reinforce one another like a "molecular velcro" (Figure 14).

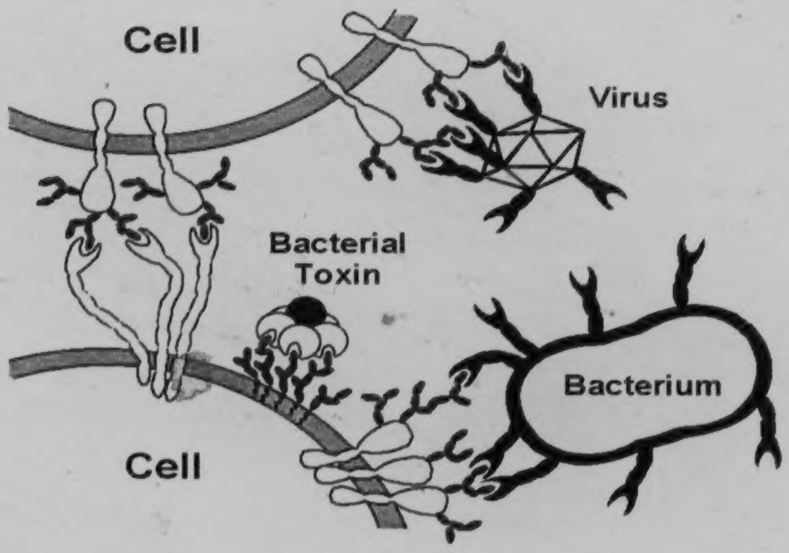

Figure 14: Multivalent carbohydrate-protein interactions In nature (Adapted from Lindhorst, 1998)

It has been shown that molecules containing many carbohydrates clustered together, bind far more strongly than those molecules, which do not possess this multivalency - a phenomena often called the glycoside cluster effect (Lindhorst *et al.*, 1998). Little is understood about the description of how antibodies recognize oligosaccharides at a molecular level, however chemists are preparing molecules containing carbohydrate clusters which can mimic natural "multiantennary carbohydrates" (Maaheimo *et al.*, 2000). The crystal structures of the cell surface LPS of *Salmonella enterica* as a dodecasaccharide and its trisaccharide fragment as well as a *Shigella flexneri* pentasaccharide fragment have been studied in complexes with Fab fragments (Bundle, Eichler, *et al.*, 1994; Vyas *et al.*, 1991).

More recently, a five-pronged sugar molecule in the shape of a starfish cluster has been found to be 1to10-million-times more effective at inhibiting *Shiga* AB5 toxic molecules than any inhibitor discovered to date (Kitov *et al.*, 2000).

1. Hypothesis and Objectives

E.coli O157:H7 has been of particular interest to many research laboratories because of its pathogenicity. The development of a rapid, sensitive and reproducible detection method could prevent further contamination and would be a most useful tool in dealing with *E. coli* O157 infection. Thus, it would be valuable to have a sensitive detection system to identify a specific epitope on the lipopolysaccharide.

Most commercially available enzyme-linked immunosorbent assays incorporate a polyclonal *E. coli* O157:H7 antibody instead of monoclonals, perhaps because of the ease and low cost of preparation of the polyclonal antibody, and the commercial unavailability or limited availability of monoclonal antibody. The challenging part of preparing a monoclonal antibody against LPS of *E. coli* O157:H7 antigen is related to the simple yet repeating chemical structure of the LPS molecule.

Anti-*E. coli* O157 monoclonal antibody will lead to the development of a highly sensitive inexpensive bacterial diagnostic as an economic alternative to more expensive PCR techniques.

Each *E. coli* cell has been estimated to have approximately 2x10^6 LPS molecules. Hence, I hypothesize that an anti-LPS MAb can efficiently form a homosandwich to avidly capture and detect whole bacteria or its shed LPS micels or vesicles in contaminated food, water and fecal samples. Using the right cocktail of antigen for stimulation of B-cells and further *in vitro* and/or *in vivo* stimulation of spleen B-cells will lead to the development of monoclonal antibody with high affinity and specificity to the LPS part of *E. coli* O157:H7. Even though monoclonal antibodies against *E. coli* O157:H7 have been previously prepared for non-commercial or limited use, there is very scarce information regarding the actual immunization protocols. Monitoring of antibody response in serum will determine the appropriate timing for hybridoma fusions.

The specific objectives of my research are:

a. Extraction of lipopolysaccharide from E. coli O157:H7 heat killed bacteria.

b. The development of monoclonal antibodies against enterohemorragic *E. coli* O157:H7.

c. The establishment of an immunofluorescent assay to detect low concentration of the pathogen.

d. The development of a sensitive homosandwich assay for whole bacteria and shed LPS of this pathogenic strain employing newly available fluorescent and chemiluminescent substrates.

The organization and flow of my research in the methods, results and discussion is shown in the chart below:

a. Isolation of LPS

↓

b. Immunization

↓

c. Humoral response measurement

↓

d. Hybridoma fusions and P124 selection

↓

e. Characterization of P124

↓

f. Epifluorescent antibody assay

↓

g. Homosandwich ELISA

↓

h. Results

↓

i. Discussion

↓

j. Conclusions

3. Materials and Methods

3. 1. Antigen Preparation

3. 1. 1. Preparation of *E. coli* O 157:H7

3.9% agar in distilled water was autoclaved for 30 minutes at 121°C and cooled in a hot water bath to 50 °C. 5% sheep blood (Faculty of Agriculture, U of A) was added aseptically; swirled and poured into Petri dishes. Plates were incubated at 37 °C overnight for quality control of sterility. One plate was used for preparing *E. coli* strain 0157 H:7 (ATCC 43895, The Global Bioresource Center™, Manassas, Va, USA). *E. coli* were plated onto agar plates supplemented with 5% whole sheep blood and incubated for 18 h. The colonies were removed from the plates and suspended in Trypticase Soy broth (Difco Laboratory, Detroit, MI, USA). A 500 mL inoculum (in four 250 mL Erlenmeyer flasks) was prepared by inoculating *E. coli* culture from plate to Trypticase soy broth media at 37 °C with agitation on a rotary shaker at 250 rpm. The next morning, the culture was exposed to 65 °C for 1 h. The cultures were checked by phase contrast microscopy. For the quality assurance of heat killed bacteria, a 0.5 mL of the culture from each flask was removed and spread evenly over the surface of a blood agar plate using a sterile plastic spreader and the dishes were incubated at 37 °C. For safety reasons, the purified *E. coli* bacteria were prepared only from heat-killed bacteria and not viable organisms. Heat killed bacteria were centrifuged (Mistral 2000) at 3500 rpm for 20 minutes and the media was replaced by sterile phosphate buffer saline (PBS) pH 7.4. The pellet was resuspended in PBS and centrifuged at 3500 rpm for 15 minutes. The washing procedure was repeated 3 times. Finally, the pellet was resuspended in 1 mL PBS and measured OD at 600nm. Total cell number was calculated as 10^8 cells/mL= 0.1 O.D.$_{600\ nm}$. The pellet was diluted with PBS to a final concentration of 10^8 bacteria in 1 mL. 1 mL aliquots of 10^8 cell/vial were prepared and stored at –20 °C for further experiments.

3. 1. 2 Sonicated *E. coli O* 157: H7

Bacteria for the whole-cell preparations were grown on 5% whole sheep blood agar plates, suspended in pyrogen free water and incubated for 1h at 65 °C. Samples were transferred to 15 mL tubes, placed into an ice bath and sonicated (maximum setting for microprobe) for 10 seconds, rested for 2 minutes, and resonicated for 10 seconds. The process was repeated 6 times. Sonicated whole cell bacteria were prepared on the day of immunization.

3. 1. 3. SDS-Polyacrylamide Gel Electrophoresis

The *E. coli* strain was analyzed by SDS-PAGE and Coomasie blue staining as follows: The *E. coli* strain was boiled in 2% SDS (Bio Rad, Richmond, Ca, USA) sample buffer for 4 min. Approximately 20 μL of sample (10^8 cells) was loaded into the 0.4% SDS / 10% polyacrylamide gel surface. Electrophoresis in a Mini-Protean II cell (Bio Rad, Richmond, Ca, USA) was conducted by applying constant voltage of 150 Volts for 45 minutes to 1 h (observing the Fast blue R staining line as a marker to terminate the electrophoresis). The gel was stained with Coomasie blue for 1 hour and destained in 25% methanol: 7% acetic acid solution.

3. 1. 4. Protein Estimation

The determination of the concentration of protein of washed *E. coli* O157:H7 cells in PBS was based on the method Bradford described in Bio-Rad Protein Assay Manual.

An aliquot of 3 mL dye reagent concentrate was diluted with 12 mL of DDI water. The acidic dye was filtered through a Whatman filter paper to remove particles. Egg albumin at 4000 μg of protein per mL was used as the protein standard. Standard protein was prepared in 8 dilutions as 4 mg/mL, 2 mg/mL, 1 mg/mL, 0.5 mg/mL, 2.5 mg/mL, 1.25 mg/mL, 0.625 mg/mL, and 0.3125 mg/mL. Duplicates of standard and *E. coli* O157:H7 cells were pipetted in 10 μL volume into separate microplate wells. Dye reagent of 200 μL volume was added into each well. The reaction mixture was placed on microplate mixer and thoroughly mixed for 3 minutes. The differential color change of the dye occurred within 5 minutes. Absorbance was quantitated at a wavelength of 590 nm on a Microplate Reader Molecular Device spectrophotometer. Plotting absorbance values versus protein concentration of egg albumin standards gave a linear standard curve and protein content of the samples was calculated.

3. 2. Lipopolysaccharide Extraction

The LPS was extracted from the *E. coli* O157:H7 cells by a modification of the method described by Westphal and Jann (1965). All saline and water used were pyrogen free. For LPS extraction, *E. coli* O157:H7 was grown in 500 mL of Trypticase Soy broth (Difco Laboratory, Detroit, MI, USA). Broth culture was grown at 37 °C for 18 h with.

Isolation of Lipopolysaccharides

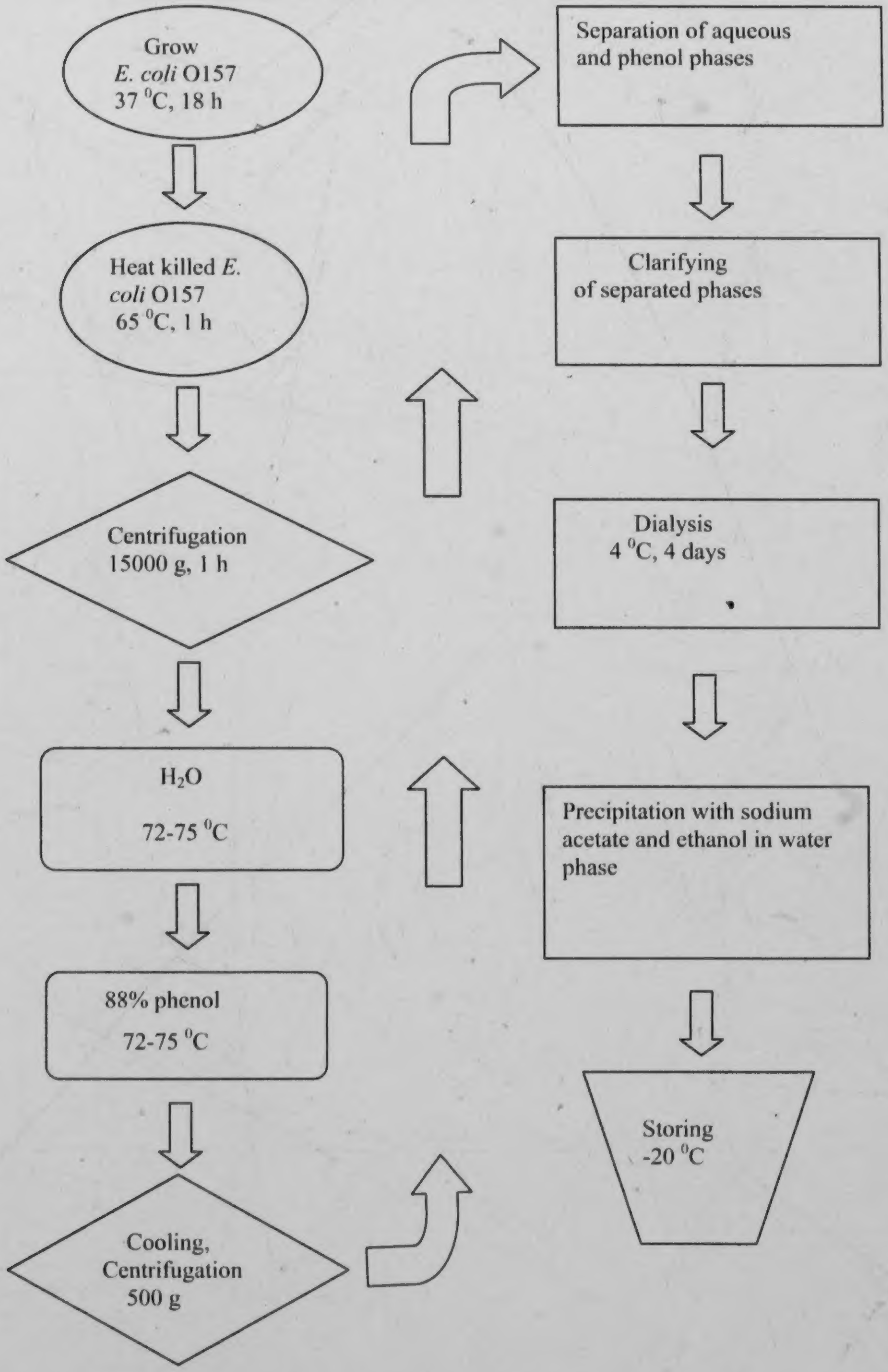
Grow
E. coli O157
37 °C, 18 h
Heat killed E. coli O157
65 °C, 1 h
Centrifugation
15000 g, 1 h
H2O
72-75 °C
88% phenol
72-75 °C
Cooling, Centrifugation
500 g
Separation of aqueous and phenol phases
Clarifying of separated phases
Dialysis
4 °C, 4 days
Precipitation with sodium acetate and ethanol in water phase
Storing
-20 °C

Figure 15: Flow diagram of the modified hot phenol-water extraction procedure

agitation on a rotary shaker at 250 rpm. The culture was exposed to 65 ^{o}C for 1 h. Cells were pelleted by centrifugation at 10 000 g for 25 minutes at 4 ^{0}C. The cell pellet was resuspended in 25 mL 0.85% NaCl and pelleted again by centrifugation. The heat-killed *E. coli* O157:H7 were washed thrice in saline, then the cells were resuspended in 10 mL pyrogen free H_2O. Ribonuclease (160 μg) and deoxyribonuclease (20μg) were added, and the mixture was stirred at 37 ^{0}C for 4 h. Proteinase K (200 μg) (GibcoBRL, Gaithersburg, MD, USA) was added, and the reaction mixture was maintained at 37 ^{0}C for 12 h. The suspension was mixed vigorously for 20 seconds, and placed uncapped in a 72-75 ^{0}C water bath. An equal volume of 88% liquid phenol (preheated to 72-75 ^{0}C) (Sigma, St. Louis, Missouri, USA) was added, and the tube was vortexed and incubated for 15 minutes in the water bath. The suspension was revortexed every few minutes during the 15-min incubation period followed by cooling to 20-23 ^{0}C on ice. After centrifugation at 500g for 10 minutes, the clear upper aqueous layer was carefully collected. The phenol-phase interface was heated to 72-75 ^{0}C, and another 10 mL of pyrogen free water (72-75 ^{0}C) was added and the process was repeated. After the phenol mixture was revortexed, the phases were once again separated by centrifugation. The two aqueous phases were combined and reheated to 72-75 ^{0}C, 5 mL of 88% phenol (72-75 ^{0}C) was added, the mixture was incubated for 15 min (72-75 ^{0}C) with regular revortexing, and the extraction process was repeated with regular revortexing. The phenol and aqueous phases were clarified by centrifugation at 10 000 g for 20 minutes at 4 ^{0}C. The aqueous and phenol phases were placed dialyzed (molecular weight cutoff 12 K-14 K) extensively in water (4 ^{0}C) for 4 days with frequent changes of the dialysate. The phenol phase containing long chain LPS was lyophilized and stored at –20 ^{0}C. The LPS in aqueous phase was precipitated with 95% ethanol (containing 0.15 g sodium acetate per 50 mL volume) in a

1:6 ratio. The aqueous phase LPS/ethanol mixture was stored at –20 ^{0}C for 12 h. This suspension was centrifuged at 7 000 g for 1 h at 4 ^{0}C and the pellet was suspended in 5 mL pyrogen free water, aliquoted and stored at –20 ^{0}C.

3. 2. 1. *Limulus* Amoebocyte Lysate (LAL) End-point Chromogenic Assay.

In vitro detection of bacterial LPS as endotoxin was determined by *Limulus* amoebocyte lysate (LAL) end-point chromogenic assay. The LAL reagents used in this method were obtained from FDA-licensed manufacturer of the kit (Associates of Cape Cod Incorporated, Falmouth, Massachussetts, USA) and were designed specially for the LAL assay. A source of LAL reagent water (LRW) was sterile water for injection obtained from University Hospital Pharmacy. Sterile glassware and disposable polystyrene plastics were used to minimize interference with the LAL test. Serial dilutions of *E. coli* O157:H7 LPS were prepared in pyrogen-free water. Control standard endotoxin (CSE) of *E. coli* O113:H10 LPS was reconstituted in pyrogen free water by vortexing for a few seconds less than 2 h before the test. Dissolved CSE contained a measured weight of endotoxin whose activity was determined from the Certificate of Analysis. Serial dilutions of the standandard were prepared in duplicates. Internal control standard endotoxin (ICSE) of LPS O157:H7 (kindly provided by Dr. Conlan, NRC, Ottawa), *Salmonella typhimurium* LPS (kindly provided by Dr. Bundle, Department of Chemistry, University of Alberta) of known concentrations were prepared in serial dilutions. Pyrochrome reagent containing an aqueous extract of amoebocyte of *Limulus polyphemus* and a chromogenic substrate was reconstituted with cold Pyrochrome buffer by gentle mixing for 7 minutes with emphasis not to create any foaming. Reconstituted Pyrochrome was kept on ice and used within an hour. 96-well sterile microplates were coated with 100 μL of each dilution of CSE, ICSE, and samples were added into the wells. For negative controls, LRW was added to six wells. A 100 μL aliquot of Pyrochrome reagent was added to each well as rapidly as possible to all samples, the plate was sealed with parafilm and mixed on a plate shaker for 40 min at RT. The optical density was measured on a Vmax kinetic microplate reader (Molecular Device Corp., California, USA) at 405 nm. The correct incubation period for color development was determined by preliminary tests for each serial dilution.

3. 2. 2. Lipopolysaccharide Analysis

SDS-PAGE was carried out as per the protocol described by Laemli, 1970. LPS samples in water were mixed with an equal volume of 0.1 M Tris-HCl buffer, pH 6.8, 2% (w/v) SDS, 20% sucrose (w/v), 1% 2-mercaptoethanol (v/v), and 0.001% bromphenol blue (w/v). The mixture was heated in a 100°C water bath for 3 minutes, and 10-20 μL samples were applied to 16% separating gel. Electrophoresis in a Mini-Protean II cell (Bio Rad, RichMond, Ca, USA) was conducted by applying constant voltage setting at 150 Volts for 1 h.

The LPS was analyzed by a modified SDS-PAGE silver stain method (Switzer *et al.*, 1979, Tsai *et al.*, 1981). The LPS was visualized by silver stain after oxidation with 0.7% periodic acid. The gel was soaked in 7% acetic acid for 7 minutes and then soaked in 200 mL of 50% methanol for 20 minutes. Intensive washing (2 times for 10 minutes) removed the excess reagents. Fixing solution was replaced with 0.7% periodic acid in 40% ethanol and oxidized for 5 minutes. Three 15-minutes washes in 1000 mL d-H_2O water were performed. The gel was treated with 20% (w/v) silver nitrate for 15 minutes, then rinsed in

200 mL d-H_2O for 5 minutes in two cycles. The gel was developed in 1% citric acid and 37% formaldehyde until the bands were usually visible after 10 minutes. Rinsing each gel with three changes of 200 mL water stopped the reaction.

3. 3. Immunization Protocol

3. 3 .1. Preparation of Antigen in Adjuvant

Freund's adjuvant consists of mineral oil containing Arlacel A as an emulsifier. The complete adjuvant (FCA) contains killed *M. tuberculosis* while the incomplete antigen (FIA) does not (Freund, 1951). FCA and FIA were purchased from Sigma Chemical Co., St. Louis, Mo. Purified heat killed *E. coli* antigen was resuspended in PBS. For primary immunization, the Freund's complete adjuvant was used. One volume of *M. tuberculosis* (FCA) was put into a 15 mL tube, chilled to 4 °C and resuspended by vortexing. An equal volume of sterile antigen solution was mixed with the FCA or the FIA oil phase in a 1:3 ratio. Antigen solution of one third of the FCA volume was drawn into a syringe with a 20 g needle and added into the oil drop by drop, while continuously vortexing.

The emulsion should be very thick. Testing for a properly prepared emulsion was conducted by placing a drop of emulsion on the surface of saline solution. A properly formed emulsified drop does not disperse over an aqueous surface.

For the subsequent immunization antigen solution of *E. coli* 0157:H7 and LPS O157:H7 were prepared in the Freund's incomplete adjuvant (FIA) emulsion and sterile PBS.

3. 3. 2 Immunization of Mice

Animal treatment and care were carried out according to the guidelines of the Health Sciences Laboratory Animal Services (HSLAS) of the University of Alberta. Five Balb/c, age 6 to 8-week-old female mice, were immunized with heat killed *E. coli* bacteria following an immunization protocol in Table 1. After the second and third immunization by intraperitoneal injection, the mouse was bled by the tail vein and their titer of anti *E. coli* O157:H7 antibodies in serum was determined by a direct binding ELISA as described in section 3. 4.

A second group of 5 mice was immunized intraperitoneally with heat killed whole cell *E. coli* bacteria (10^{10} -10^{6} CFU), and a third group of 5 mice was pretreated with *E. coli* heat killed bacteria and further immunized with LPS O157:H7 NRC standard. The immunization protocol for the best B-cell producing anti-LPS O157:H7 antibodies was carried as described in Table 2. For evaluation of immunogenicity, test bleeds were collected. Mice were chosen for hybridoma fusion when an IgG antibody level increased about 10 folds.

The mice were immunized under aseptic conditions as per protocol by an intrasplenic injection of the antigen described in section 3. 3. 3.

Time (Days)	Dose of Antigen (CFU)	Adjuvant	Volume (mL)	Route
0	10^8	FCA	0.2	IP
8	10^8	FICA	0.2	IP
10	10^9	PBS	0.2	IP
12	10^8	PBS	0.2	IS
15	**Cell Fusion**			

Table 1: Short- term immunization protocol. Five Balb/c mice were immunized with heat killed *E. coli* O157:H7 bacteria in complete Freund's adjuvant (FCA) with 10^8 cells (CFU), followed by immunization with Freund's incomplete adjuvant (FICA). The third subsequent intraperitoneal injection (IP) of antigen in phosphate buffer saline (PBS) was followed by an intrasplenic injection (IS) three days prior to hybridoma fusion.

Time (Days)	**Dose of Antigen**	**Adjuvant**	**Volume (mL)**	**Route**
0	10^8 CFU	FCA	0.2	IP
12	5 μg LPS	FICA	0.2	IP
26	5 μg LPS	PBS	0.2	IP
36	2.5 μg LPS	PBS	0.2	IP
48	10^8 CFU + 5 μg LPS	PBS	0.2	IS
54	**Cell Fusion**			

Table 2: Long-term immunization protocol. Balb/c mouse was immunized with heat killed *E. coli* O157:H7 bacteria in complete Freund's adjuvant (FCA), followed by immunization with lipopolysaccharide (NRC) in Freund's incomplete adjuvant (FICA), and subsequent intraperitoneal injection (IP) of antigen in phosphate buffer saline (PBS). An intrasplenic injection (IS) was administered under aseptic conditions.

3. 3. 3. Intrasplenic Injection of the Antigen

The last immunization of mice prior to splenic cell fusion was administered by an intrasplenic injection of the antigen (*E. coli* heat killed bacteria or LPS). The mouse was anaesthetized by inhaling Methfane (Spitz, 1986) and placed on its right side, under aseptic conditions, in a biohazard hood. A skin incision 1-1.5 cm long was made below the rib cage on the left side and the skin was separated from muscles. The lower pole of the spleen was lifted with forceps and a 27-gauge needle fitted to a 1 mL syringe was inserted deeply into spleen. The concentration of antigen was either 10^8 heat killed *E. coli* O157 or a combination of 10^8 cells of *E. coli* with 5 μg of LPS in 200 μL of PBS. After injection of antigen, the spleen was pushed back in to the cavity, and the skin edges closed with several stiches. The mouse was resuscitated from the anaesthesia with oxygen and kept under observation for several hours. On the third day (in one case on the 6 th day) following the intrasplenic injection the mouse was euthanized and the speen was obtained for the hybridoma fusion.

3. 4. Humoral Immune Response

For evaluation of immunogenicity, test bleeds were collected to monitor serum antibody levels. In addition, blood was collected from each mouse after sacrifice, and the antibody titer was measured by direct Enzyme-Linked Immunosorbent Assay (ELISA).

Briefly, 96 well microtiter plates (Nunc, Naperville, IL, USA) were coated with 10^8 heat killed bacteria or 2 μL/well LPS O157 antigen and incubated for 15 h at 4 ^{0}C, and at 37 ^{0}C for 2 h. The plates were washed and incubated with 200 μL of 2% dialyzed BSA-T for 1 h at 37 ^{0}C to block nonspecific binding. The plates were washed three times with 250 μL PBS containing 0.1% Tween-20 and 2% horse serum (PBST-HS) (or multicycle washing in excess volume of washing buffer). After washing, 100 μL of the serum samples at several dilutions (1:10- 1:100 000) were added to the wells and incubated for 3 h at 37 °C. The wells were washed three times with PBST-HS. Subsequently, 100 μL of a 1:10 000 dilution of goat anti-mouse IgG (whole molecule) HRPO conjugate or 1:10 000 dilution of goat anti-mouse IgM (u chain specific) peroxidase conjugate (Sigma, St. Louis, Mo.) were separately added and incubated for 1 h at 37 °C. The plates were washed three times with 250 μL PBST-HS and 100 μL of TMB peroxidase substrate (1:1) was added as a chromogen (Kirkegaard & Perry Laboratories, Gaithersburg, MD). Absorbance values at 650 nm were measured in a Vmax kinetic microplate reader (Molecular Device Corp., California, USA) after 5, 15, 30 and 45 minutes of color development.

3. 4. 1. Stability of Serum Sample

The tail vein blood sample was collected into an eppendorf tube and kept for 3 h at 4 °C. The serum was separated by centrifuge at 14 000 g for 1 min. Serum with the highest ELISA activity against *E. coli* O157 was diluted in PBS and kept at –20 °C as the internal positive control sample. The vial was thawn and frozen several times within a 4 months period and ELISA activity was determined.

3. 5. Hybridoma Development

3. 5. 1. The Fusion Partner Cell Line

One week prior to cell fusion, the SP 2/0- Ag 14 myeloma cell line was grown in complete 20% DMEM medium at a density of 5×10^4 cells/mL in a tissue culture flask at 37 ^{0}C in 5% CO_2 atmosphere with 98% relative humidity (a container filled with sterile distilled water). Gradually, the concentration of FBS in DMEM was lowered according to the strength and growth of the myeloma cells. A minimum of 40 million viable myeloma cells was needed for fusion. Cell viability was determined on the day of fusion by the Trypan Blue exclusion method. Cell viability at the time of fusion was greater than 95%. The myeloma cells were washed 3 times with DMEM media at 700 g for 7 minutes (MISTRAL, 200). To ensure that the cells collected are in log phase of growth, the cell density was adjusted to 2×10^5 cells/mL the day before the fusion by adding fresh medium.

The SP 2/0 cell line was chosen as the fusion partner for immune spleen cells because of its good growth rate, the efficiency with which hybridomas are obtained after fusion, HAT sensitivity, and most importantly because it does not synthesize or secrete any immunoglobulin heavy or light chains.

3. 5. 2. Viability Cell Count

Trypan Blue is a stain that is actively excluded from viable cells, but which readily enters and stains dead cells. A non-viable cell will have a blue cytoplasm while a viable cell will have a clear cytoplasm. Therefore, the cells, which are blue, are dead. The difference between the total number of cells and the number of dead cells would be the number of viable cells in a given aliquot of the culture.

The suspension culture was gently swirled to distribute the cells evenly. A 900 μL sample was aseptically removed from the cell culture into an eppendorf tube. Four parts of 0.2% (w/v) stock Trypan Blue were diluted with 1 part of 5x PBS; 100 μL of the diluted dye was added to the sample (1x dilution). The cell suspension was mixed gently and immediately placed on the hematocytometer.

The percentage of viable cells were calculated as follows:

$$\text{Viable cells (\%)} = \frac{\text{Number of viable cells} \times 100\ \%}{\text{Total cells}}$$

$$\text{Total number of cells/ml (cells/cm3)} = \frac{\text{mean number of cells}}{0.1\ \text{mm}^3\ \text{volume}} \times 10^4$$

3. 5. 3. Hybridoma Cell Fusion

The selection of fused hybridoma cells is achieved by culturing the cells in media that support the growth of hybridomas but prevent the growth of the indefinitely dividing myeloma cells. The hybridoma cells are selected against hypoxanthine, aminopterin, and thymidine (HAT) medium (Sigma Chemical Co., St. Louis, MO, USA) where hybridoma cells survive due to hypoxanthine phosphoribosyl transferase gene of the somatic spleen cells as described in section 1. 10. 2.

The spleen was aseptically removed from immunized mice and teased using a 5 mL syringe with a 21 gauge needle to flush the cells in and out to get single cell suspension with DMEM (Sigma Chemical Co., St. Louis, MO, USA) media. The spleen cells were washed 3 times with DMEM by sedimentation at 700 g for 7 minutes. Finally, the total spleen cell count was determined using a hematocytometer. The spleen cells were mixed with myeloma cells in ratio of 5:1 and suspended in 5 mL DMEM. The mixed cells were washed three times with 15 mL serum free DMEM media. After the final wash, the supernatant was completely aspirated from the mixed-cell pellet.

To the pellet, 0.5 mL of 50% 37 °C polyethylene glycol (PEG) solution (Sigma, St. Louis, MO) was added drop by drop with constant gentle stirring of the pellet over a period of 1 minute. The high osmotic pressure generated by the 50 % PEG results in membrane instability, and fusion occurs (Hui *et al.*, 1999). Following 1-minute incubation at 37 °C, the cells were pelleted by centrifugation at 500 g for 5 minutes. The supernatant was completely removed and cells were suspended in 15 mL of 20% FBS (Gibco BRL, Gaithersburg, MD, USA) in DMEM with 2mM glutamine-penicillin-streptomycin solution (Gibco BRL, Gaithersburg, MD, USA). After washing three times, the fused cells were gently resuspended with 5 mL DMEM media with 20% FBS. Leaving the cells in clumps, 2 mM glutamine-penicillin-streptomycin (GPS) solution, and 10 % Origen Hybridoma Cloning Factor (OHCF) (Igen International, Inc. Gaithersburg, MD USA) was added to the pellet. The fused cells were gently transferred to a sterile polystyrene petri dish with additional 15 mL of DMEM-20/OHCF-10 media and incubated at 37 °C in 5% CO_2 for 30 minutes. After 30 minutes, cells and incubation medium were transferred into a polystyrene tissue culture flask with 85 mL of DMEM - 20/OHCF-10 media and mixed slowly.

The suspended, unstable newly formed hybrid cells (150 μL/well) were gently dispensed into four 96-well flat-bottom assay plates (Nunc, Naperville, IL, USA) and incubated as before for 24 h. Following the 24 h incubation, 100 μL of HAT (2x)/ OPI (Oxalacetic acid sodium pyrovate bovine insulin) (Sigma Chemical Co., St. Louis, MO, USA) media was added to each well and the hybridomas were incubated further for three days. Three days after the fusion, 50 μL of supernatant was aspired from each well and fresh DMEM media with 20% FBS, 2mM GPS, HAT (2x) was added to each well. A minimum of two additions of fresh HAT media was performed on different occasions.

Hybridoma growth in all wells over the next 2 to 4 weeks was observed daily with an inverted microscope. When the wells turned yellow or growing clones showed confluence by examination under microscope, they were ready to be tested for the desired antibody. Hybridomas were usually visible ten days after fusion. The hybridomas in the microtitre wells were allowed to build a higher concentration of antibody in the culture over a period of several days. Thus, it was important to examine the plates frequently to avoid the wells becoming yellow and overly acidic and causing the death of hybridomas. Hybridomas with strong binding affinity were evaluated by direct ELISA and selected for cell cloning with limited dilution.

3. 5. 4. Limited Dilution Cell Cloning

Limited dilution is a cell cloning procedure where the cells are plated at a very low concentration to ensure that the reclones would have come from a single cell.

Cloning was performed by counting the fused cells in the wells of the 24 well plates. Two different cell densities of 100 and 40 cells in DMEM media with 20% FBS, 2mM glutamine-penicillin-streptomycin (GPS) solution, and 10% Origen Hybridoma Cloning Factor (OHCF) media per each 96 well microtiter plates were prepared for following recloning. These plates were incubated in a humidified incubator at 37 ^{0}C with an atmosphere of 5 % CO_2. Three days after the limited dilution cloning, 50 μL of supernatant was removed from each well by aspiration and fresh DMEM media with 20% FBS, and 2mM GPS was added to each well and was then incubated until hybrid clones appeared in the microtiter wells. The clones showing ELISA values 3-5 times higher than that of the negative control were considered for further expansion. The entire content of the original well from the 96 well microtiter plate was transferred to a well in the 24-well plate. The clones were prescreened for antibody production using the direct ELISA method. Hybridoma was cloned a second time by limited dilution with 40 cells per 96 well plate until >90% cloning efficiency was reached, establishing the monoclonality and stability of the cell line.

Clone efficiency is calculated as follows:

$$\text{Cloning Efficiency (\%)} = \frac{\text{Number of antigen positive clones} \times 100}{\text{Number of total clones in the recloning step.}}$$

3. 5. 5. Cell Banks

Hybridoma of both the parental and the cloned lines were stored in liquid nitrogen. Selected hybridoma clones were expanded and cells at the logarithmic phase were washed and suspended in freezing media (90% FBS + 10% DMSO) at 5 x 10^6 cells/vial in sterile polypropylene screw cap freezing vials (Corning Glass Works) while kept on ice. The vials were stored in the cryobox at –80 °C and transferred to liquid nitrogen. Each hybridoma line was maintained in 6 well tissue culture plates until the frozen cell lines were successfully re-established in culture after being stored frozen for a minimum of one week in liquid nitrogen. Once a cloned hybridoma line showed good growth in DMEM media with 20 % FBS, its parent hybridoma line was discarded.

3. 6. Production of Monoclonal Antibodies

3. 6. 1. Tissue Culture Flask Production of Monoclonal Antibodies

Once a stable monoclonal antibody-producing hybridoma cell line had been isolated and safely stored, large amounts of monoclonal antibodies were produced *in vitro*. The hybridoma cell line grown in DMEM-20 was transferred from the 6-well polystyrene tissue culture plate into a 75 cm^3 tissue culture flask. Antibody secreting hybridomas were first grown in complete DMEM-20 media and the supernatant was screened for adequate production of the desired antibody. The cells were gradually adapted to a medium containing 5 % fetal bovine serum (Gibco BRL, Gaithersburg, MD, USA). Tissue culture flasks were inspected with an inverted microscope to determine cell viability and density. In addition, the hybridomas were monitored for contamination. Depending on the viability and cell numbers, a portion of the cell mass was reintroduced into a tissue culture flask with fresh medium. The supernatant of each cloned hybridoma in exponential growth phase was harvested every three to four days in sterile 50-mL conical centrifuge tubes. The cells were centrifuged at 900 g for 7 minutes at RT. The supernatant was then collected and the pellets discarded. The supernatant was aliquoted and stored under sterile conditions for immediate testing at 4 ^{0}C, in storage at –20 ^{0}C.

3. 6. 2. Large Scale Production of Monoclonal Antibody Supernatant

Bioreactors are often used in monoclonal antibody production as alternative systems to the ascites method for milligram quantity production.

The cells were cultured in the complete DMEM media with 20% FBS. Supernatant was screened for adequate production of the antibody. When it was certain the antibody secretion was reasonable for the time in culture, the cells were adapted to DMEM media with 10% FBS. Approximately 50 million cells were diluted with 90 mL of nutrient medium supplemented with a high concentration of 10% FBS. The cells were cultured in the tissue culture flask with 30 mL DMEM-10 medium. Four days after innoculation, the cell compartment was harvested, the cells counted, and their viability determined. Depending on the viability, the cell mass was diluted in 200 mL DMEM media with 10% FBS and added toa SiCulture Bag™. (Medtronic Perfusion Systems, MN, USA). The new culture device was filled with 2 L of DMEM media with 10% FBS. To prevent any contamination, the surface of the bag was treated with 95% alcohol. The bag was incubated at 37 ^{0}C with 5% CO_2 and high humidity until the cell viability decreased to 20%. After an incubation period of 4 weeks, the bag was removed from the incubator, hung on a ring stand with the ports facing downwards. The contents of the bag were harvested into a 50 mL conical centrifuge tubes. The supernatant was collected as described in section 3. 6. 1.

3. 6. 3. Antibody Purification

3. 6. 3. 1. Ammonium Sulfate Precipitation

All classes of antibody precipitate as non-denatured salt complexes when treated with 40-50% wt/vol. of saturated ammonium sulfate at iced cold temperature. The high concentration of ammonium sulfate causes protein precipitation by competing for the solvent water molecules. Water molecules bind to the proteins through hydrogen bonds, keeping the protein in solution. If the ammonium sulfate attracts enough water away from the proteins, the proteins lose their water of solvation and come out of solution. Normally, this occurs without gross structural changes in the protein and is a gentle method of removing protein from solution (England *et al.*, 1990).

High-grade solid ammonium sulfate (Sigma Chemical Co., St. Louis. MO, USA) was slowly added with stirring to 1 L of harvested supernatant in ice bath. (Stirring is necessary to avoid localized high concentrations of ammonium sulfate that may cause precipitation of non-immunoglobulins). To achieve the desired 50% saturation percentage, the stirred protein solution was kept on ice during the precipitation process. Stirring of precipitated proteins continued for 12 h at 4 ^{0}C. The solution was then centrifuged for 30 minutes at 900 g. The pellet was resuspended in 25mL of PBS and dialyzed (molecular weight cutoff 12 K -14 K) for 72 h against 1 L of PBS at 4 ^{0}C with 4 changes of dialyzing buffer. To obtain concentrated antibody solution, the contents in dialyzed bag were dehydrated with solid PEG 80 (Sigma Chemical Co., St. Louis, MO, USA) at 4^{0}C to reduce the final volume to 5 mL.

3. 6. 3. 1 Polyethylene Glycol Precipitation

The initial ammonium sulfate precipitation of antibody was replaced by high molecular weight polyethylene glycol 6000 that could be used above zero temperatures with little risk of protein denaturation. Polyethylene glycol is an ethylene oxide polymer, which are strong Lewis bases and form hydrogen bonds with water molecules. The proteins lose their water of solvation and come out of solution resulting in their precipitation (Anastassiadon, and Biziagos, 1996, Technical Bulletin JRH Biosciences, 2003).

One volume of 25% polyethylene glycol (PEG) 6000 (Sigma, St. Louise, Mo) solution in normal saline was mixed with 1 volume of collected supernatant. The mixture was stirred at 4 ^{0}C (ice bath) for 30 min. The precipitate was collected by centrifugation at 14 000 g for 10 min. All the supernatant solution was removed and the pellet was centrifuged at 14 000 g for 10 min to express the trapped PEG. The pellet was dissolved in 25 mL of PBS and dialyzed (molecular weight cutoff 12 K -14 K) for 72 h against 1 L of PBS dialyzing buffer at 4 ^{0}C with four changes of PBS.

3. 6. 3. 3. Protein G-Sepharose Affinity Chromatography

Protein G is a 60 kDa protein found on the cell walls of *Staphylococcus aureus*, which binds to the Fc region of an antibody. Protein G has a high affinity to antibody at pH 5. The bound protein can be eluted from the column at pH 2.8 (Akerstrom and Bjorck, 1986).

The dialyzed protein precipitate was diluted with 0.1 M sodium acetate at pH 5.0 in 1:2 ratio for the supernatant harvested from the tissue culture flasks and at 1:10 ratio for the supernatant obtained from the SiCulture Bag. A protein G (Amersham Pharmacia Biotech) 5 mL volume column was equilibrated in 0.1 M sodium acetate, at pH 5, and 4 ^{0}C. The diluted antibody solution was layered onto the resin bed at flow rate of 1 mL/min. The column was washed with 0.1 M sodium acetate at a flow rate of 1 mL/min. The bound antibody was eluted with freshly prepared 0.1 M glycine hydrochloride (pH 2.8) at a flow rate of 0.5 mL/min into 10 mL tubes. To minimize the effect of low pH on the collected protein, 0.1 M Tris-based buffer was added into each tube. Preliminary test determined the amount of 0.1 M Tris-buffer to be added into 1 mL glycine hydrochloride for a final pH of 7.5. Approximately 1mL fractions were collected and the absorbance at 280 nm was determined to calculate the amount of antibodies in each tube.

SDS-PAGE of the purified monoclonal antibody P124 was carried out as described in section 3. 1. 3.

3. 6. 3. 4. Protein G-Sepharose Affinity Chromatography Second Cycle

The Protein G column 5 mL volume was equilibrated in 0.1 M sodium acetate, at pH 5, and 4 ^{0}C for 4 h. The unbound fractions collected from the first cycle of Protein G affinity chromatography were pooled and loaded onto the resin bed at flow rate of 1 mL/min. The column was washed with 0.1 M sodium acetate at a flow rate of 1mL/min. The bound antibody was eluted with freshly prepared 0.1 M glycine hydrochloride (pH 2.8) at a flow rate of 0.5 mL/min into 10 mL tubes as described in section 3. 6. 3. 3. The bound and unbound fractions were collected and evaluated for ELISA activity against *E. coli* O157.

3. 7. Conjugation of Long Chain Biotin NHS Ester to Antibodies

The streptavidin-biotin system has been used as a versatile nonisotopic detection system for sandwich enzyme immunoassay. Streptavidin is a nonglycosylated 60 kDa protein isolated from the yeast *Streptomyces avidinii.* Four biotin molecules can be conjugated to one molecule of tetrameric streptavidin. Thus, the antibody exposes its conjugated biotin ligands as a universal, highly reactive, nonisotopic, primary probe. Streptavidin indirectly bridges biotin with a secondary probe acting as a most versatile catalyst to any system containing hydrogen peroxide. Thus, the sensitivity of the two-step sandwich assay will be increased without affecting binding ability of the antibody to the antigen. Further enhancement of the sensitivity of the system can be achieved by eliminating any steric effects between biotin and streptavidin by using the biotin ester a "long chain arm" which ensures that the protein-linked biotin is freely accessible to bind to the streptavidin conjugated horseradish peroxidase (Marqulies, 1998; Lisanti, 1995).

3. 7. 1. Biotinylation of Whole Supernatant

Biotinylation of 2 mL of each supernatant sample was performed by adding 200 µL of 16 mg/mL biotin LC-NHS ester in DMSO (100 µL + 900 µL 0.1 M $NaHCO_3$). The 15 mL conical centrifuge tube was wrapped in foil and incubated at RT for 12 h with gentle shaking. To terminate the reaction, 100 µL of 10 mg/mL glycine in PBS was added. Excess biotinylated glycine was removed by dialyzing the solution against 4 L PBS pH 7.2 at 4 ^{0}C for 24 h.

3. 7. 2. Biotinylation of Monoclonal Antibody

Purified monoclonal antibody of a known concentration was covalently bound to long chain NHS-Biotin (N-Hydroxysuccinimidbiotin, Sigma Chemical Co., St. Louis, MO, USA) in a 8:1 ratio (1 mg antibody: 125 mg of NHS-Biotin). A 15 mL conical centrifuge tube was wrapped in foil and incubated at RT for 4 h with gentle shaking. To terminate the reaction, 41 µg of glycine in solid form was added to the reaction mixture. The excess biotinylated glycine was removed by dialyzing the solution against 4L PBS pH 7.2 at 4 ^{0}C for 48 h.

3. 8. Indirect Fluorescent Antibody Assay

The chamber slides were coated with 1 mL of live bacteria (10^8 CFU) harvested from TSB growing media and allowed to air dry before they were fixed in cold (-20 ^{0}C) acetone for 10 minutes. The cells were washed with ice-cold PBS. One mL of blocking agent was added and the chambers were incubated for 0.5 h at 37 ^{0}C. The chamber slides were aspirated and washed 3 times with ice-cold PBS. Two different concentrations (2 μg, 5 μg) of purified monoclonal antibody P124 in 2 mL dilution buffer were added to slides; and dilution buffer was also added to one slide as a negative control. After incubation at 37 ^{0}C for 2 h, the slides were washed with excess of ice-cold PBS-T-HS washing buffer. After washing 3 times with ice-cold PBS, goat anti-mouse IgG conjugated to fluorescein isothiocyanate (FITC) (Kirkegaard & Perry Laboratories Inc., Gaithersburg) was added to the chamber in 1:50 dilution in PBS. The chambers were wrapped in aluminum foil to prevent photobleaching and incubated for 30 minutes at 4 ^{0}C. The chambers were washed 5 times with ice-cold (4 ^{0}C) PBS in the dark and then fixed with 1% formaldehyde fixative reagent for 30 minutes at 4 ^{0}C. Excess formaldehyde was removed and the chambers were gently washed 5 times with ice-cold PBS. Mounting solution was prepared as 90% glycerol and 2.5% DAPCO in PBS. The chamber system was disassembled, the slides were mounted with a drop of mounting solution and covered using 24 x 60 mm No.1 Fisherbrand coverglass. The slides were viewed with

Zeiss LSM 510 microscope under excitation/emission at 488/530 nm by using a 40 objective magnification.

3. 9. Enzyme Immunoassay

3. 9. 1. Screening Primary Hybridoma Supernatants by Direct Enzyme-Linked Immunosorbent Assay (ELISA)

The aim of screening all wells was to examine which hybridoma secreted the antibody. Two experiments were conducted. In one, a 96 well ELISA plate was coated with 100 µL heat killed *E. coli* (10^8 CFU per well) suspended in PBS at pH 7.2. In the second experiment the *E.coli* antigen was suspended into carbonate buffer at pH 9.3. Non-covalent attachment of the antigen to the microtiter plate was allowed at 4 °C for 15 h. After washing the wells three times with 200 µL of PBS - 0.1% Tween 20 (PBS-T), the non- specific binding sites were blocked with 200 µL/well of 2% dialyzed bovine serum albumin dissolved in PBS-T. The coated wells were incubated for 2 h at room temperature using a microtiter mixer. Three washes with excess volume of PBS-T-HS were used to remove free blocking reagent from the well. One hundred µL of diluted supernatant (1:2 ratio) was added in duplicate. Two wells of an irrelevant supernatant and DMEM media were added to function as control samples. Once again, the wells were blotted dry and washed three times with excess PBS-T and 100 µL of goat anti-mouse-IgG-HRPO conjugate in 1% BSA at dilution 1:1000 was added and incubated for 1 h at RT followed by another 3 times washing procedure. Just prior to the next procedure, 3, 3', 5, 5'-tetramethyl benzidine (TMB, KPL, USA) peroxidase substrate was mixed with H_2O_2 (1:1). The TMB/ H_2O_2 100 µL mixture was added to each well and the plate was then agitated for 5 minutes. The optical density was determined on a microplate reader at 650 nm in 10-minute intervals within 30 minutes of color development.

3. 9. 2. Two-step Homosandwich Immunoassay

The ELISA sandwich is one the most versatile ELISA assays. Only multivalent antigens with different or repeating epitopes may be detected in this assays (Figure 16).

Purified anti-LPS monoclonal antibody (designated as P124) at 2 µg/well in PBS-T-BSA was immobilized on a 96-well polystyrene microplate and incubated for 2 h at 37 ^{0}C. The plates were washed with 200 µL of PBS-T and blocked with 150 µL of blocking agent (2% dialyzed BSA-Tween 20, pH 7.2) then incubated for 2 h at 37 ^{0}C. After washing with excess volume of PBS-T-HS 6 times, various amounts of *E. coli* O157:H7 or LPS O157 in 100 µL volume were added and incubated overnight at 4 ^{o}C. After washing with excess volume of PBS-T-HS, 100 µL of biotinylated antibodies were added into each row of the microtiter plate and incubated for 2 h at 37 ^{0}C overnight. The plates were washed 3 times with PBS-T and incubated with 100 µL of streptavidin-HRPO conjugate of 1:10000 dilutions for 2 h at RT. Once again, the plates were then washed with PBS-T and the color was developed by incubating the wells with 100 µL of TMB (colorimetric), QuantaBlu™ (fluorescence), Pico™ , and Femto^{Tm} (chemiluminescence) substrate. The absorbance was measured at 650 nm after 10 minutes and repeated over the next 45 minutes every 10 minutes (colorimetric). Chemiluminescence or fluorescence was determined within the first 5 minutes at the appropriate wavelength (fluorometric, chemiluminescence) on a Molecular Devices Gemini Spectramax XS microplate reader. A positive control and negative control of irrelevant hybridoma supernatant along with the samples of interest were measured in duplicate.

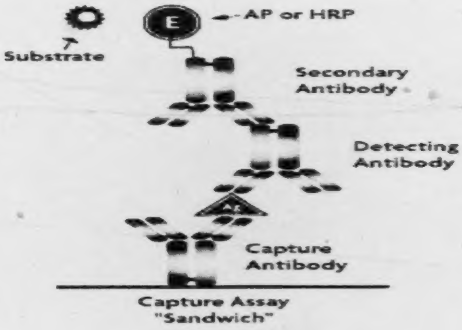
AP or HRP
E
Substrate
Secondary Antibody
Detecting Antibody
Capture Antibody
Capture Assay "Sandwich"

Figure 16: Schematic of homosandwich ELISA

(Adapted from diagram by Kirlegaard & Perry Laboratories Inc., 2002)

4. Results

4. 1. Preparation of the LPS Antigen

Crude LPS was obtained from the pelleted cells washed with PBS by hot-phenol extraction and ultracentrifugation. The purest LPS obtained from the pelleted material of the PBS washed cells, digested enzymatically with Proteinase K prior to hot-phenol extraction and ultracentrifugation (Figure 15). LPS O157 was found both in the phenol and water phase of a phenol-water extract. Proteinase K treatment apparently did not affect the level of reactivity of the epitope with the MAb in the ELISA testing (Figure 17, Figure 18).

The LPS of *Escherichia coli* O157:H7 cells (50 g wet weight) extracted with hot-phenol and precipitated with ethanol/sodium acetate gave LPS from both the water phase (10 mg) and the phenol phase (30 mg). The LPS samples from both the water and phenol phase were recognized by monoclonal antibodies for LPS O157:H7 (Figure 18).

SDS-PAGE of the water phase LPS and the phenol soluble S-LPS was conducted to further characterize the samples. Figure 19 shows the faint ladder like banding pattern of LPS O157 on an SDS -polyacrylamide gel. The separation of O-chain LPS produced bands in the 43 kDa region in phenol extract appears evident when compared to previous studies (Perry 1986; Chart, 1993). The core bands of rough LPS in aqueous phase were observed in 12 to 16 kDa region. The aqueous sample possessed a large proportion of R-type LPS core structures presumably uncapped by O-polysaccharide. The sensitivity of this assay is approximately 1μg of LPS O157.

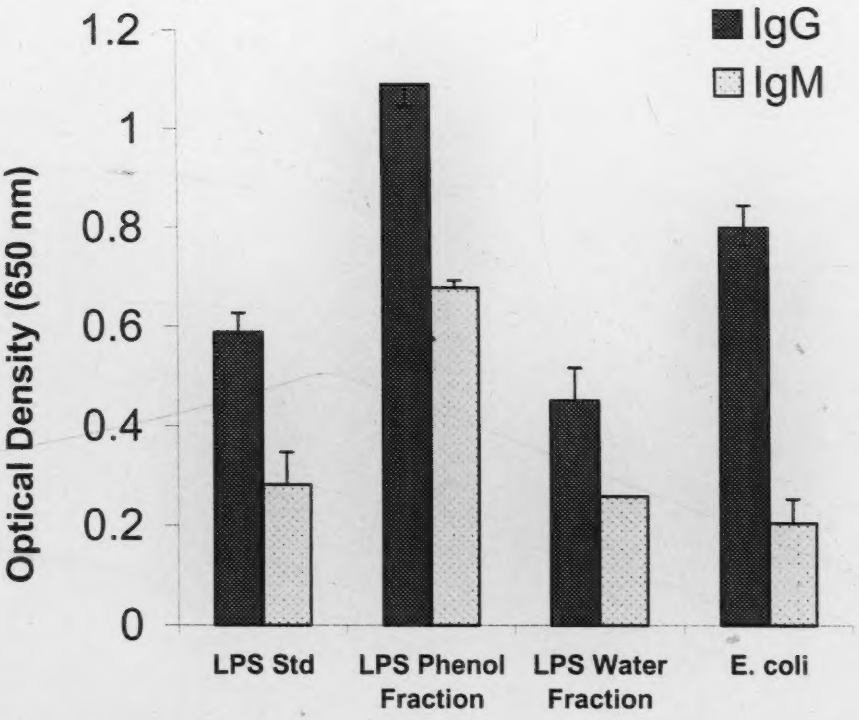

Figure 17: ELISA activity of the LPS-containing extracts. Each LPS was extracted by the hot phenol-water procedure. The LPS was treated with deoxyribonuclease, ribonuclease (100 µg/mL), and proteinase K (20 µg/mL). The solution was dialyzed against distilled water until free of phenol (tested with ferric chloride for elimination of the purple color). Each ELISA plate was coated with extracted LPS at 10 µg/well. LPS (NRC LPS O157:H7) was coated at 0.2 µg/well. *E. coli* O157:H7 heat killed bacteria in 50 mM bicarbonate buffer (pH 9.6) were coated in 10^8 CFU/well. Anti-LPS O157:H7 serum in PBS-0.001% Tween 20-0.1% BSA and pH 7.4 (1:10 dilution) were incubated for 1 h at 37 ^{0}C, then for 15 h at 4 ^{0}C with the analyte. After washing in PBS-0.01% Tween 20-0.01% horse serum (HS) at (PBS-T-HS) pH 7.2, the plates were incubated with horseradish peroxidase-labeled goat anti-mouse immunoglobulin G and immunoglobulin M (separately in 1:10 000 dilutions) at 37 ^{0}C for 1hr and developed for 30 min with TMB: H_2O_2, and the absorbance at 650 nm was recorded.

Error bars correspond to SD calculated on duplicates (LPS) and triplicates (*E. coli*).

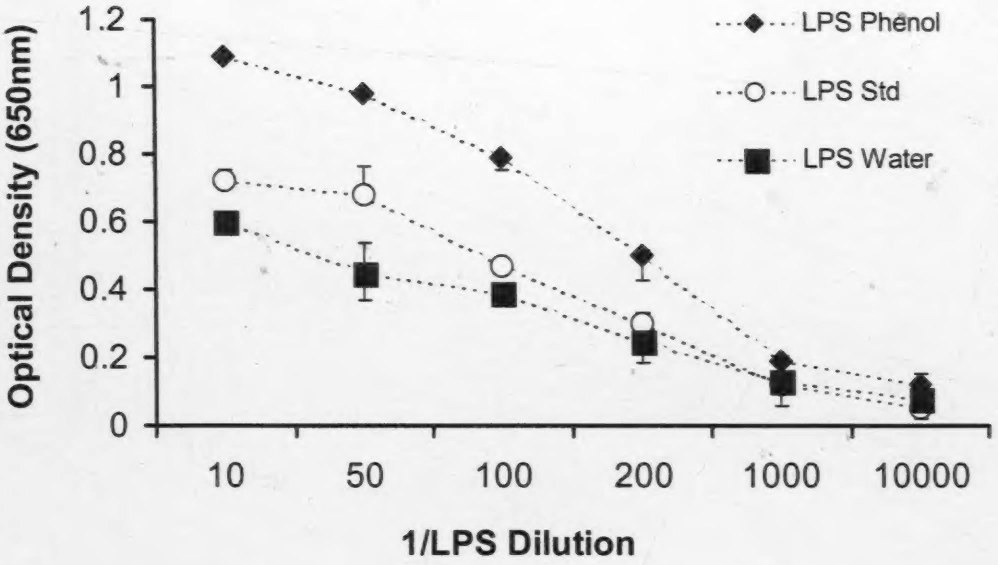

Figure 18: Reactivity of LPS. The isolated *E. coli* O157:H7 LPS was recognized by anti-LPS O157:H7 antibodies with high titers for LPS O157:H7. The relative concentration of isolated LPS was estimated by performing a standard ELISA using varying amounts of purified LPS. Plates were coated with extracted LPS at 10 μg/well and LPS (NRC LPS O157:H7) at 0.2 μg/well and their various dilutions followed by 2 h incubation at 37 ^{0}C. A 150μL aliquot of blocking buffer (2% dialyzed BSA (DBSA)-0.001% Tween-20) was added to each well and was then incubated for 15 h at 4 ^{0}C. Plates were washed with PBS-T-HS buffer. A 100 μL aliquot of anti-LPS O157:H7 antibodies (20 μg/mL) in dilution buffer was added into 87 wells. A 100 μl aliquot of dilution buffer and a 100 μL aliquot of positive control 13B8 monoclonal antibody (Dr. Westerman, USDA, Montana, USA) was added. Following the wash step, the second HRPO-conjugated antibody was added, the color was developed with TMB and the optical density at 650 nm was measured. Standard deviations are indicated as error bars. When not visible, bars indicating the standard error of the mean are smaller than the symbol.

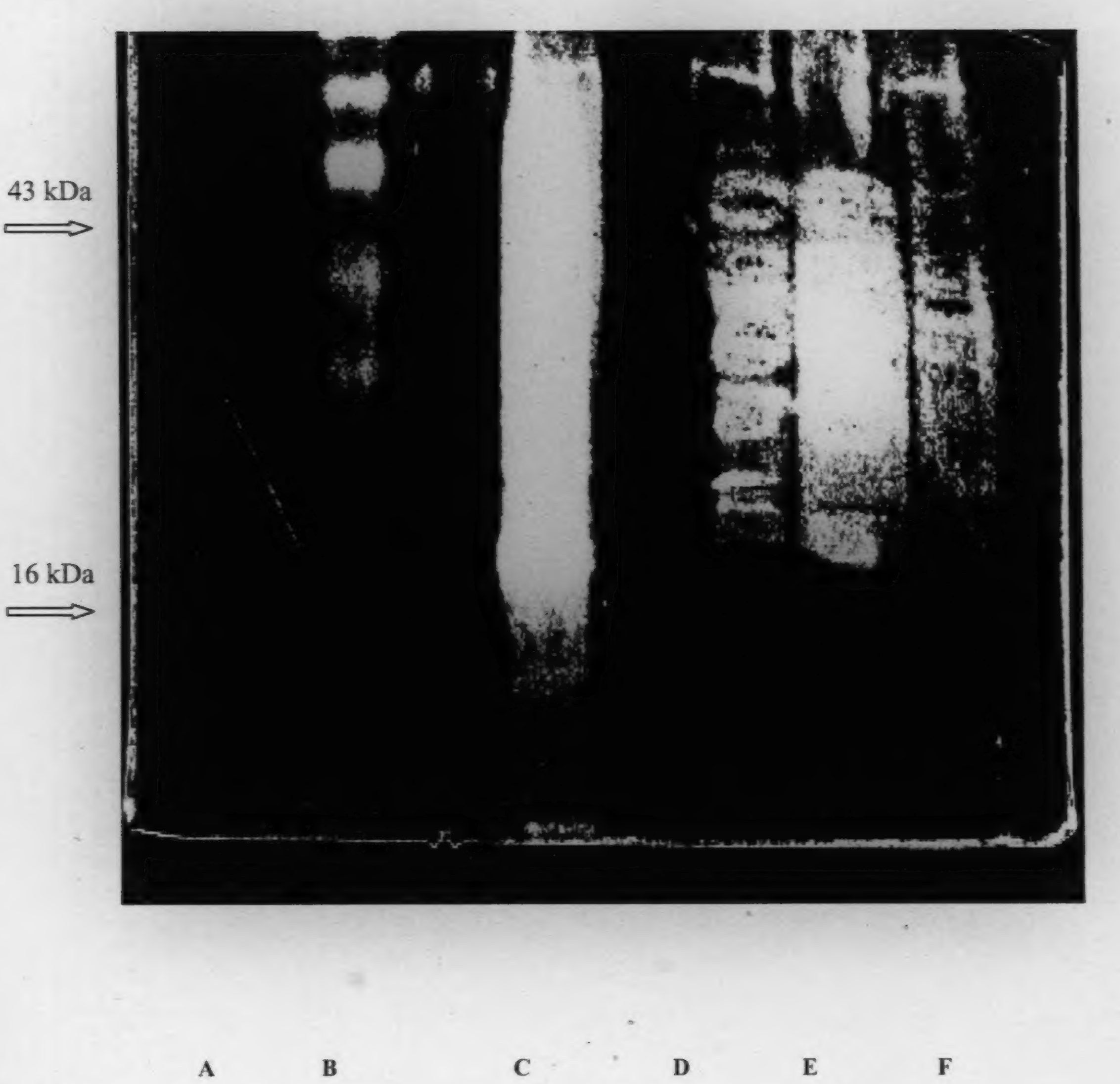

Figure 19: SDS-Polyacrylamide gel electrophoresis of LPS. The samples were electrophoresed at 150 V for 1 h on 15% SDS-PAGE incorporating 4 M urea into gel. The LPS was visualized by the silver stain with 0.7 % periodic acid oxidization. Samples A= 0.1 μg LPS O157 NRC is not visualized; B= 1 μg of LPS O30 shows bands of O-LPS in 43 kDa region; C= 1 μg water extract of LPS O157 with the band around 16 kDa (lower part); D, E, F, are samples of LPS O157 phenol extract in various concentrations.

4. 2. Estimation of Endotoxin in LPS O157 by *Limulus* Amoebocyte Lysate Assay

The biological activity of the LPS O157 extracted from *E. coli* O157:H7 was evaluated by activation of the clotting enzyme of *Limulus* amoebocyte lysate (LAL). The amount of endotoxin in LPS O157, estimated by *Limulus* amoebocyte lysate assay, was about 5.3 x 10^3 endotoxin units per µg. *E. coli* O113:H10 was used as a Control Standard Endotoxin (CSE). *E. coli* O113:H10 activity was standardized to a Reference Standard Endotoxin of *E. coli*-5 (RSE: EC-5) chosen by the Food and Drug Administration. It should be pointed out that the LAL-method does not represent an absolute LPS measurement (Heederik, 1991; Jones, 2001). The data presented corresponds to the portion of endotoxins that are biologically active and available to the assay (Figure 19).

4. 3. Immune Response in Mice by Short-term and Long-term Immunization Protocol

Three groups of mice were immunized with *E. coli* O157:H7 bacteria. Five mice (Group 1) were immunized in a short-term immunization protocol with a constant concentration *E. coli* O157:H7 heat killed bacteria. Mice sera tested in the ELISA exhibited low to moderate levels of O157:H7 antibodies (Table 1, Table 3).

The second group of 5 mice was immunized in long-term immunization protocol with various concentrations of *E. coli* O157:H7 heat killed bacteria. The third group of 5 mice was immunized in a long-term protocol with the cocktail of antigen. Table 1 and Table 2 summarize the standard immunization protocol and its modification, respectively.

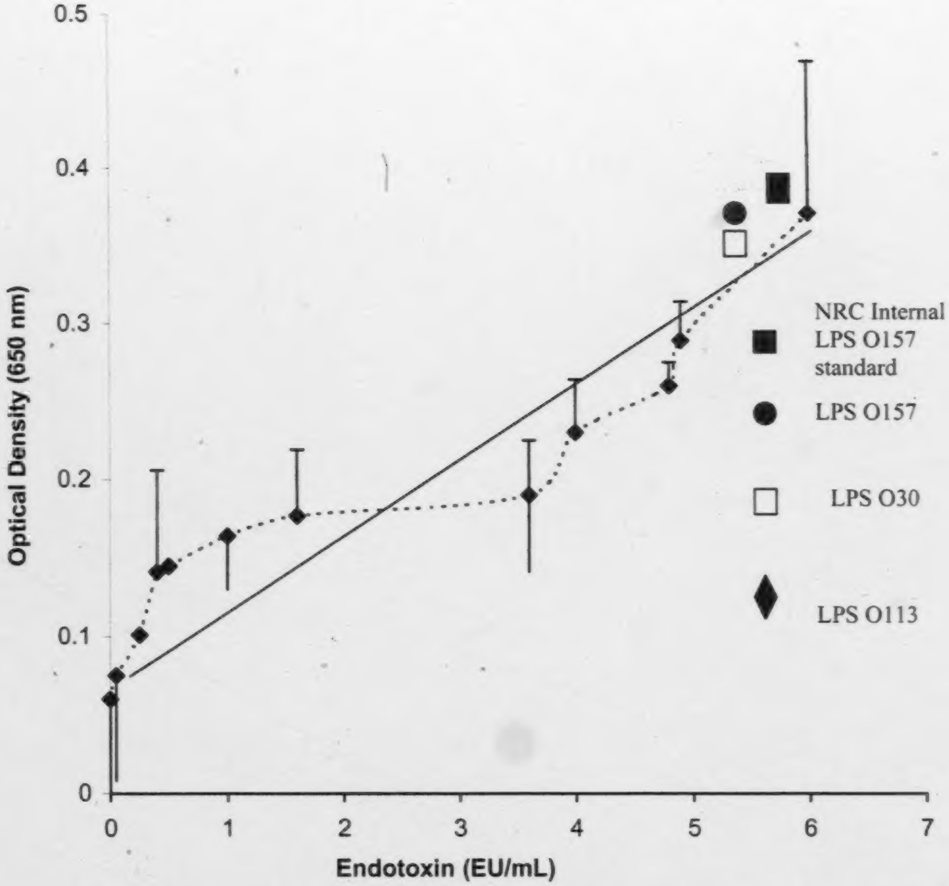

Figure 20: Standard curve for endotoxin estimation of bacterial LPS. Detection of bacterial LPS activity as endotoxin was determined by *Limulus* amebocyte lysate (LAL) end-point chromogenic assay. The portion of endotoxins that are biologically available to the LAL assay is expected to represent a relative toxicity measure. Dissolved *E. coli* O113:H10 LPS contained a measured weight of endotoxin whose activity was given in the Certificate of analysis (Associates of Cape Cod Incorporated) provided. Internal control standard endotoxin (ICSE) of *E. coli* O157:H7 LPS (Dr.Conlan, NRC), *Salmonella typhimurium* LPS O30 (Dr. Bundle, U of A), of known concentrations (50 ng/mL), along with my preparations of *E. coli* O157:H7 LPS, were prepared in pyrogen free water. 100 L of CSE, ICSE, and samples of *E. coli* O157:H7 LPS were coated on a sterile 96-well microplate. LAL reagent water was added to nine wells as a negative control. A 100 L aliquot of reconstituted LAL reagent (coagulogen+ Pyrochrome^{TM} chromogenic substrate) was then added, incubated for 45 min as per standard protocol, and then absorption at 405 nm was measured. One EU equals approximately 0.1 ng endotoxin/ml of diluted analyte. All standards and samples were assayed in quadruplicate, and experiments were carried out at 25 ^{0}C. Values represent the mean and error bars correspond to SD. The mean optical density of the negative control being performed in replicates of nine was 0.06.

Group	N (mice)	Dose of Antigen (CFU, µg)	IgG
I	5	10^8 EC*	0.193 +/- 0.03
II-A	2	10^7- 10^9 EC	0.291 +/- 0.09
II-B	2	10^8 –10^{10} EC	0.371 +/- 0.04
II-C	1	10^{10} EC	0.283 +/- 0.07
III-A	2	2.5 LPS	0.202 +/- 0.01
III-B	1	10^8 EC, 5 LPS	0.422 +/- 0.03
III-C	1	10 LPS	Died
III-D	1	10^7 EC, 10 LPS	0.398 +/- 0.02

Table 3: Antibody response to various concentrations of antigen. Three separate groups of mice (five mice in each group) were tested for IgG response following the administration of various concentrations of antigen. Mice in Groups I and Group II-C were administered heat killed *E. coli* O157:H7 bacteria in equal concentrations for each immunization (EC*). Mice B and D in Group III were pretreated with heat killed *E. coli* O157:H7 bacteria (CFU) prior to immunization with LPS O157 (µg). Serum samples were collected on the 15th day (Group I), and on the 16th day (Group II and Group III) after the first immunization and IgG levels were measured by ELISA. The values are the mean and standard deviation of three OD_{650} measurements, with the blanks subtracted.

4. 3. 1. IgM and IgG Response

Using an ELISA method capable of differentiating IgM from IgG, the class of antibodies present in the sera during the immunization period was determined. The IgM and IgG response of one mouse to *E. coli* O157:H7 LPS is shown in Figure 21. Slight increases of IgM antibodies followed the first two injections. The peak of IgM serum level was seen at 33 days and appeared to decline subsequently. The peak IgG antibodies were also seen around 33 days and were more than IgM levels on day 48.

4. 3. 2. Comparative Response to LPS of *E. coli* O157:H7

Representative titration curves of serum samples obtained from the immunization experiments with heat killed *E. coli* O157:H7, heat killed and sonicated *E. coli* O157:H7 and O157 LPS are shown in Figure 22. To compare antibody responses, endpoint O157 antibody titers were determined at the highest dilution giving an $O.D_{650nm}$ value greater than the mean of the $O.D_{650nm}$ value of 0.2. Immunization of mice with 10^6 CFU of *E. coli* O157:H7 induced little change in their titers. In contrast, the greatest difference in the titer was seen in the ELISA, in which the *E. coli* O157:H7 were heat killed and sonicated along with purified O157:H7 LPS by long-term immunization. This protocol evoked titers approximately 100-fold higher than those of the *E. coli* bacteria alone. This preparation of antigen was effective in eliciting high titers of IgG antibody, which recognizes native cell surface of *E. coli* O157:H7 and the LPS O157 determinant.

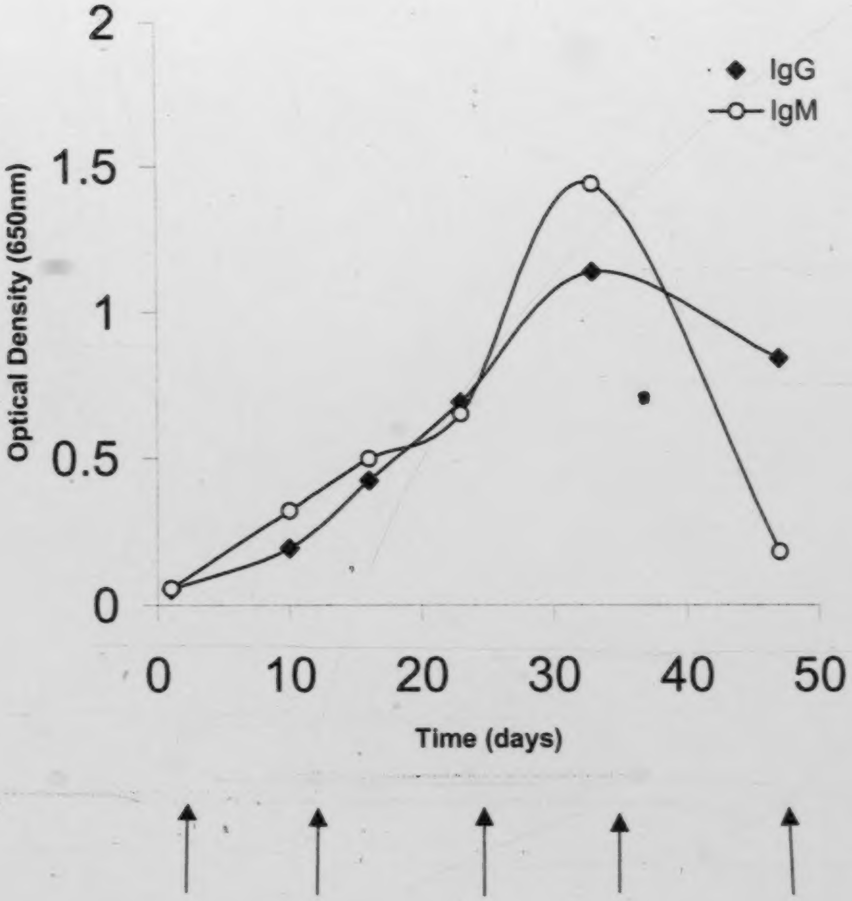

Figure 21: Antibody (IgG, IgM) response in serum of mouse immunized with heat killed *E. coli* O157:H7 cells and LPS. Antibody levels were measured by ELISA using the whole cells as an antigen and expressed as ELISA values (absorbance at 650 nm) for serum at 1:50 dilution. The peak of antibody response was 33 days after the initial immunization. Further antigen stimulation decreased the IgM titer and had minimal effect on the IgG response. The values represent the mean of the optical density at 650 nm of an IgM–ELISA and IgG-ELISA performed in duplicate.

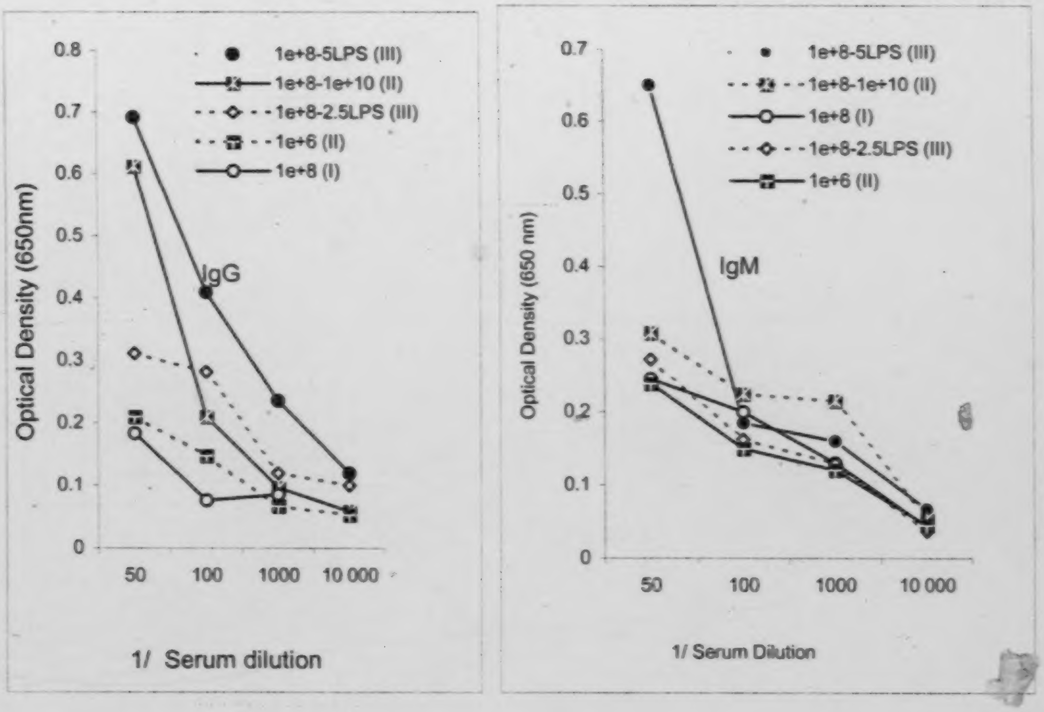

Figure 22: Serum titers of anti-*E. coli* O157 antibodies: Three separate groups of mice (5 for each group) were immunized in either short-term (Group I) or long-term immunization protocol (Group II, and Group III). Microplates were coated with 10^8 CFU/well heat killed *E. coli* O157:H7. A 100 μL of diluted serum in 1:50,1:100, 1:1000, and 1:10 000 dilution buffer were added into each well, incubated for 2 h at 37 ^{0}C and washed. The bound anti-O157:H7 was detected by goat anti-mouse IgG labeled with HRPO (1:10 000) and TMB substrate at 650 nm. Each point represents the mean of duplicates above background. 10^6 CFU *E. coli.* O157:H7 in Group II (1e+6 (II)), and 10^8 CFU of *E. coli* O157:H7 in Group I (1e+8 (I)) induced little change in their antibodies level over those following the second injection with 10^9 CFU of heat killed *E. coli* O157:H7 in Group II (1e+8 – 1e+10 (II)). In contrast, all of the mice in Group III immunized with the cocktail of 10^8 heat killed and sonicated *E. coli* O157:H7 and 2.5 (or 5) μg of LPS O157:H7 (1e+8-2.5(5) LPS (III)) elicited antibodies. The differences in IgM titers were in the same trend as seen with the IgG titers.

Although the differences in IgM titers were not of the same magnitude as seen with the IgG titers, the trend follows a well-known pattern of IgM and IgG response (Figure 22).

The mice with strongest response to the immunogen were chosen as the candidates for the hybridoma fusion.

4. 3. 3. Mitogen-induced Proliferation of Spleen Cells.

In order to maximize B-cell reactivity to LPS, two experiments were conducted with the spleen cells primed with a cocktail of LPS antigen by long-term immunization of Balb/c mice.

In one, isolated spleen cells were stimulated with 10 μg/mL and 25 μg/mL O157 LPS *in vitro.* Mitogen stimulated spleen B-cells in 100 μL at 10^6 cells/well were incubated 48 h and 72 h at 37 ^{0}C and 5% CO_2. *In vitro* LPS stimulation did not improve the proliferation of B-cells producing anti-O157 LPS antibodies.

The second, *in vivo* stimulation of B-cells was carried out with 1 and 2.5 μg LPS in PBS by intrasplenic injections and the spleens were removed 120 h after stimulation. Mice were observed for any adverse effects to the antigen. The initial lethargy was overcome within a day. The size of the stimulated spleen was increased compared to non-pathological conditions (about 2 times).

4. 4. Stability of Polyclonal Antibodies

Aliquots of serum samples were stored at –20 ^{0}C, thawed and frozen several times. Measuring the O.D. at 650 nm measured the ELISA reactivity. The first two cycles were tested at 1:100 dilution and the next three were at 1:1000 dilution. There was apparently no loss of anti-O157 LPS response by freezing and thawing of the two dilutions (Table 4).

4. 5. Selection of Hybridoma

Monoclonal antibodies were generated by standard hybridoma fusion methods with several modifications. The aim of this work was to produce MAb against *E. coli* O157:H7 by immunizing mice with heat-killed bacteria of *E. coli* O157:H7. However, in spite of giving the mice several doses of this antigen in a short immunization protocol (Table 1), they either responded very poorly or not at all, suggesting that the time and the doses given to the mice had to be modified. Female 6-8 week old Balb/c mice were immunized in a modified long-term immunization protocol (Table 2). Mouse monoclonal antibodies to the LPS of *E. coli* serotype O157:H7 were prepared by the hybridoma technique (Kohler and Milstein, 1975). Briefly, the splenocytes were harvested and fused with SP2/0 myeloma cells. Origen Hybridoma Cloning Factor (OHCF), 10% Origen, was used to enhance the number of hybridomas which survive during the HAT selection and increases the number of cells which can secrete the antibody. The emergence of clones in plates with growth medium supplement was increased by 15% compared to those plates without the addition of HCF. The clones (SP2/0 and spleen cells O157 LPS) were screened by ELISA.

Thawing Cycles	Day	Absorbance at 650 nm Dilution Buffer	Absorbance at 650 nm Positive serum sample
1	1	0.103	1.631
2	68	0.106	1.564
3	73	0.086	1.057
4	85	0.051	1.012
5	112	0.057	1.012

Table 4: Stability of Polyclonal Antibodies. Aliquots of serum samples were stored at –20 ^{0}C, thawed, and frozen several times within a 4 months period. Microplates were coated with 10^8 heat killed *E. coli* O157:H7 CFU/well. A 100μL aliquot of diluted serum in 1:100 (thawing 1, and 2), and 1:1000 (thawing 3, 4, 5) dilution buffer were added to each well, incubated for 2 h at 37 ^{0}C and then washed. The bound anti-O157:H7 was detected by goat anti-mouse IgG labeled with HRPO (1:10 000) and TMB substrate at 650 nm. Each point represents the mean of duplicate samples.

On the basis of ELISA screening, heat killed cells were initially used and LPS positivity was subsequently confirmed using purified LPS O157 (Figure 23). The activity of antibody secreting cells against the LPS of *E. coli* O157:H7 was analyzed in 768 clones. Primary screening of hybridoma supernatants employes repeated analyses of heat-killed *E. coli* O157:H7 or LPS O157 to preferentially select positive antibody secreting clones. In most cases only hybridomas whose supernatants showed strong or moderate LPS reactivity patterns were retained for recloning and extended specificity analyses. In the first screen, 32 primary clones producing specific antibody were identified. The remaining clones were negative to *E. coli* O157:H7 or showed low binding with LPS O157. Results in Table 5 show the ELISA value of only the 32 strongly positive primary clones.

However, when the corresponding clones were tested for LPS O157:H7, it was discovered that 17 of the 32 *E. coli* O157:H7 whole cell positive clones did not bind or had a low binding density with purified LPS O157:H7. It is likely that these clones are more specific to other epitopes. Table 6 summarizes the results of analyses on the clones identified as being strongly positive to both heat-killed *E. coli* O157 and purified LPS O157. Furthermore, the supernatant from our collection that was strongly positive to LPS O157 by ELISA was tested for IgG and IgM selective primary clones (Table 6). The three strongest clones producing IgG and the three strongest clones producing IgM were selected for recloning by serial dilution.

The recloning was performed at two different densities of clones at 10 cells/mL and 4 cells/mL as described in methods and materials in section 3. 5. 4. The first subcloning of P124 resulted in a satisfactory cloning efficiency at 4 cells/mL; only those cells were considered for further studies. 10% OHCF was employed to increase the cell viability for stressed cells and to enhance their growth. Each primary positive clone was recloned into five 96-well plates. The positive clones appeared in 121 wells, resulting in an appropriately 63% increase in the isolation rate. After subcloning, eight cell lines were recovered, and from these, three were finally selected on the basis of the specificity of the secreted antibody. Table 7 summarizes the results of the analyses on the positive clones after the first recloning. The ELISA values of only the strongly positive clones compared to the negative control are shown in Table 6 (O D $_{650\ nm}$ > 0.6 (*E. coli* O157:H7)).

A second recloning was done using P124.2. After 10 days, 528 clones were evaluated for specificity to heat killed *E. coli* O157:H7 bacteria by ELISA. The final selection of clones was determined by O157 LPS-ELISA elimination tests. The second recloning of P124.2 resulted in 100% cloning efficiency. Results are summarized in Table 8 and Figure 24.

ELISA screening with both the purified LPS prepared in our lab and O157:H7-LPS standard established the O-antigen specificity of the antibodies. The relative potencies of the isolated MAb specific to O157:H7 epitopes were tested in several dilutions. Figure 24 shows a representative titration of MAb P124. Using an arbitrary optical density cut-off of 0.2 and an endpoint titer approximately 1:10 000, the subclone 1F10- 2A2-H5-2 (P124.B2) was chosen as the final antibody-producing clone for further studies.

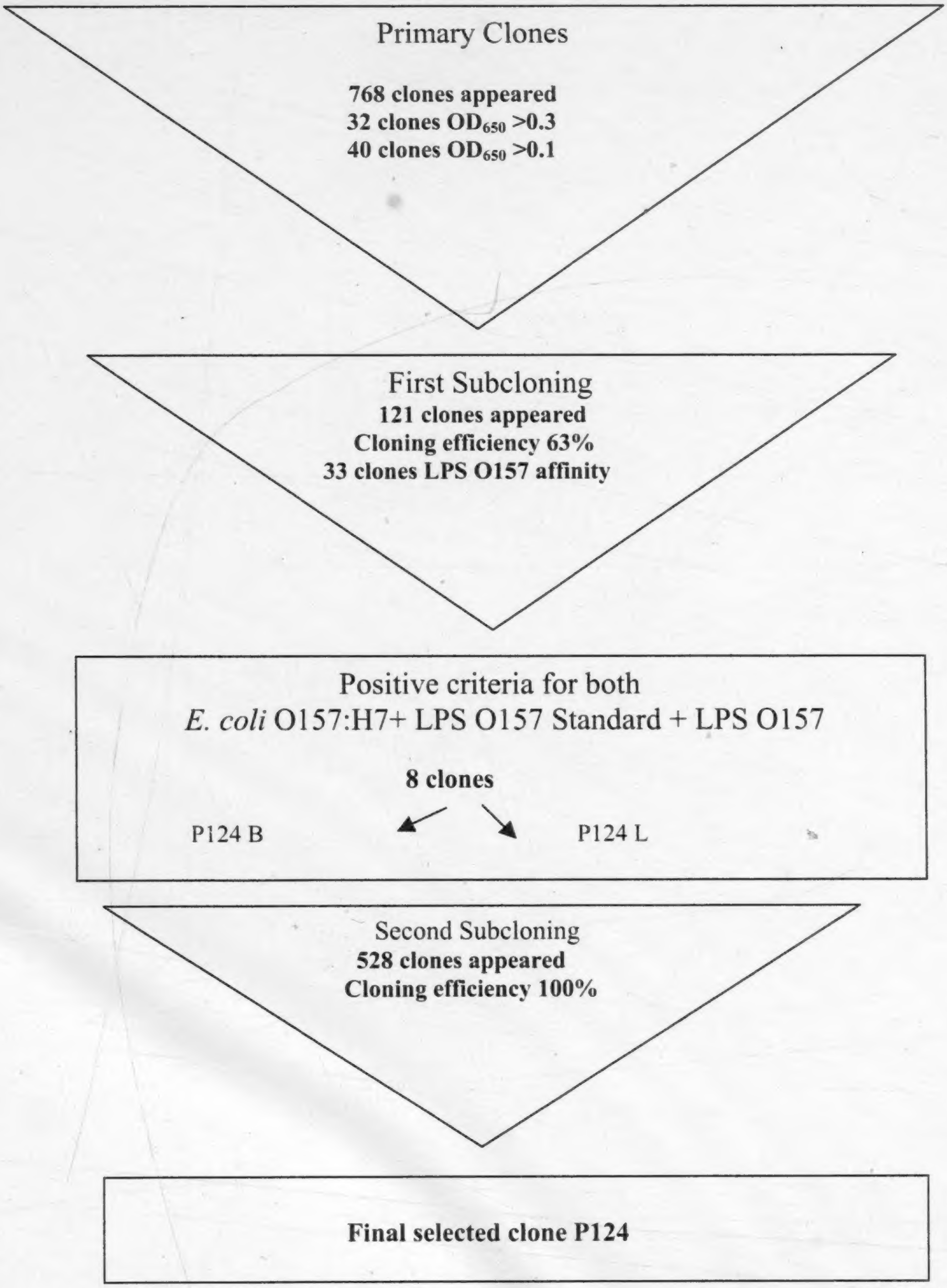
Primary Clones
768 clones appeared
32 clones OD650 >0.3
40 clones OD650 >0.1
First Subcloning
121 clones appeared
Cloning efficiency 63%
33 clones LPS O157 affinity
Positive criteria for both
E. coli O157:H7+ LPS O157 Standard + LPS O157
8 clones
P124 B
P124 L
Second Subcloning
528 clones appeared
Cloning efficiency 100%
Final selected clone P124

Figure 23: Hybridomas selection strategy and results

Cell Line	Day 7	Day 9	Day 12	Cell Line	Day 7	Day 9	Day 12
1C3	0.411	0.809	0.783	**4E 6**	0.291	1.049	1.125
1C9	0.189	0.945	0.839	**4F10**	0.475	0.623	0.713
1E6	0.478	0.891	0.926	**4G12**	0.325	0.435	0.415
1E7	0.101	0.516	0.479	**5A7**	0.396	0.532	0.546
1E 8	0.135	0.311	0.258	**5B7**	0.148	1.173	0.894
1E9	0.116	0.523	0.579	**6E6**	0.208	0.457	0.462
1 F 6	0.486	0.597	0.581	**7G1**	0.117	0.391	0.379
1F10	1.648	1.987	1.925	**7G2**	0.106	0.328	0.346
1F11	0.08	1.194	0.864	**7G3**	0.153	0.334	0.357
1F12	0.152	0.521	0.603	**7G12**	0.358	0.907	0.897
1G 5	0.136	0.803	0.837	**7H12**	0.248	0.536	0.521
1G 6	0.213	0.897	0.746	**8E5**	0.147	0.662	0.597
1G 7	0.153	0.378	0.398	**8F3**	0.538	0.464	0.492
1G10	0.827	1.037	1.236	**8F7**	0.095	0.807	0.752
3E12	0.613	0.712	0.825	**8G2**	0.258	0.319	0.361
3H11	0.394	0.325	0.315	**8G11**	0.139	0.333	0.385

Table 5: Primary hybridoma cell lines screening. The supernatants from the different clones were checked for anti-LPS O157:H7 antibodies by a direct ELISA on the seventh day after hybridoma fusion. The binding of antibodies was detected by HRPO conjugated goat anti-mouse IgG. TMB substrate was used to identify strong positive clones. The optical density at 650 nm was evaluated for 768 clones. The positive clones (32 clones) were retested after being transferred to 24-well plates on the ninth day and 6-well plates on the twelfth day. 140 clones were considered positive ($OD_{650} > 0.1$). The negative control showed a mean (n=3) OD_{650} of 0.069, 0.101, and 0.086 (day 7, 9, and 12 respectively). OD_{650} of screening Day 9 and 12 is the mean of duplicate samples.

Cell Line	**IgG (*E.coli* O157:H7)**	**IgG (LPS O157 standard)**	**IgM (*E.coli* O157:H7**	**IgM (LPS O157 standard)**
1 F 10	0.672 (0.02)	0.706 (0.05)	0.112 (0.02)	0.083 (0.09)
1 G 10	0.845 (0.03)	0.526 (0.02)	0.079 (0.05)	0.091 (0.04)
7 G 12	0.907 (0.09)	0.841 (0.09)	0.066 (0.03)	0.050 (0.06)
4 E 6	1.049 (0.11)	0.793 (0.02)	0.425 (0.03)	0. 539 (0.04)
1 G 6	0.389 (0.09)	0.275 (0.05)	0.957 (0.02)	0.835 (0.09)
7 G 3	0.334 (0.09)	0.253 (0.03)	0.821 (0.05)	0.573 (0.02)
8 G 2	0.205 (0.04)	0.178 (0.03)	0.499 (0.09)	0.258 (0.05)

Table 6: Elimination process for primary hybridoma cell lines secreting anti-LPS O157:H7 immunoglobulins by direct ELISA. The positive clones of primary hybridoma were transferred to 24-well plates. Cell culture supernatant of 32 clones (1:2 dilutions) was tested against 10^8 CFU/well *E. coli* O157:H7 heat killed bacteria and 0.02μg/well LPS O157:H7 (NRC) immobilized solid phase. Each clone was tested with goat anti-mouse IgG and goat anti-mouse IgM (B) labeled with HRPO. The OD_{650} are the mean of duplicates with their standard deviations.

Cell Line	*E .coli* O157:H7	LPS NRC	Cell Line	*E. coli* O157:H7	LPS NRC
1F10-1C3	1.62	0.163	1F10-2C1	0.836	0.145
1F10-1D1	1.682	0.189	1F10-2C3 **(P124.4)**	1.632	0.165
1F10-1E1	1.088	0.179	1F10-2C6	0.909	0.082
1F10-1G7 **(P124.1)**	1.391	0.211	1F10-2C8 **(P124.5)**	0.926	0.249
1F10-1H1	1.507	0.199	1F10-2D1	1.675	0.091
1F10-2A2 **(P124.2)**	1.355	0.515	1F10-2C10 **(P124.6)**	1.696	0.244
1F10-2A3	0.823	0.181	1F10-2D9 **(P124.7)**	1.611	0.168
1F10-2A5	0.877	0.155	1F10-2E1	1.173	0.155
1F10-2A10 **(P124.3)**	1.576	0.166	1F10-2G7 **(P124.8)**	0.959	0.167
1F10-2B4	1.735	0.111	1F10-E10	1.444	0.187
1F10-2B5	1.742	0.112	1F10-2F2	1.31	0.123
1F10-2B6	0.948	0.097	1F10-2F5	1.587	0.111
1F10-2B7	1.138	0.106	1F10-2F8	0.918	0.259
1F10-2B9	1.263	0.178	1F10-2H2	0.817	0.158
1F10-2B10	1.335	0.131	1F10- D10	0.835	0.173

Table 7: ELISA for screening anti-LPS O157:H7 antibodies after the first recloning. On the seventh day after subcloning of the primary hybridoma, the supernatants from different clones were checked by a direct binding *E. coli* O157:H7 ELISA. The positive cell lines were retested for LPS O157 epitopes after they were transferred to 24-well culture plates. Eight clones (P124.1-P124.8) were selected for second recloning by limited dilution. Further tests of the eight selected clones were carried out under P124.B and P124.L. The results are presented as the mean of the OD at 650 nm given by the dilution of the supernatant (1:20) in duplicates.

Cell Line	***E. coli* O157:H7**	**LPS (NRC)**	**LPS O157**
1F10-2A2 D10-2 (P124.B1)	1.505	0.804	1.001
1F10-2A2 H5-2 (P124.B2)	1.139	1.038	1.125
1F10-2A2 E7-2 (P124.B3)	1.154	0.886	1.252
1F10-2A2 F10-2	1.267	1.19	0.837
1F10-2A2 C10-2	1.369	0.751	0.984
1F10-2A2 D11-2	1.072	1.195	1.066
1F10-2C3 H9-2 (P124.B4)	1.115	0.866	1.148
1F10-2C3 E8-2	1.081	0.738	1.056
1F10-2C3 H9-2	1.147	1.083	1.121
1F10-2G7 D9-1 (P124.B5)	1.187	1.125	1.087
1F10-2G7 D6-1	1.123	0.991	1.149
1F10-2G7 A10-2 (P124.B6)	1.322	1.096	1.099
1F10-2G7 D5-2	1.226	1.09	1.085
1F10-2G7 E6-2 (P124.B7)	1.253	1.16	1.09

Table 8: ELISA reactivity after second recloning. Diluted supernatant (1:2) of 582 clones was tested after the seventh day of the second recloning. OD_{650} >0.3 measured in all 96 well microplates (100% positive). Fifteen clones of the highest OD_{650} were transferred into 24-well plate and retested with LPS O157 NRC standard and LPS O157 extracted from *E. coli* O157:H7 bacteria by direct ELISA. (P124.B1 was subcloned from P124.2).

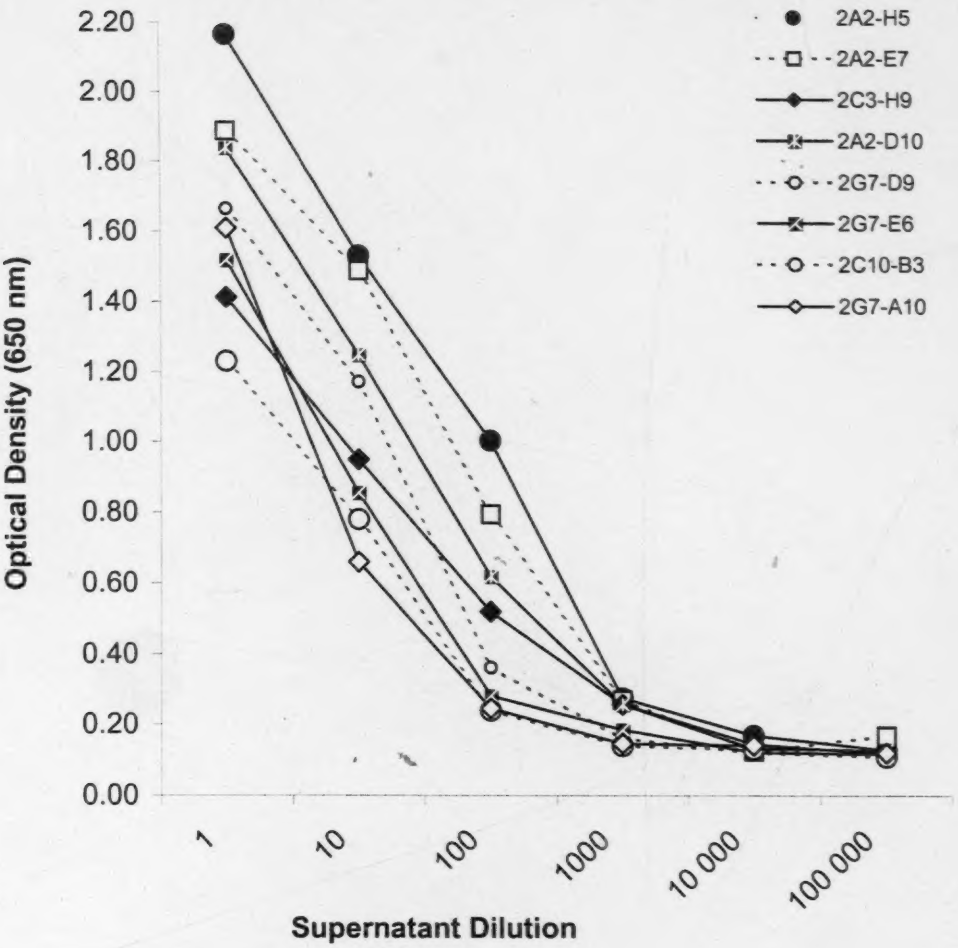

Figure 24: Titration of supernatant containing MAb from subcloned cell lines of 1F10-2A2, 1F10-2C3, 1F10-2C10, and 1F10-2G7. Seven clones were transferred into 6-well plates and retested for concentration of monoclonal antibodies in the supernatants. The supernatant of each subcloned cell line (in 1:10, 1:100, 1:1000, 1:10 000, and 1:100 000 dilution buffer) was titrated against 10^8 CFU/well of *E. coli* O157:H7. The ELISA values presented are the mean of the optical density in duplicate subtracted from the background value at 650 nm. The background reactivity (optical density), determined using PBS instead of supernatant, was 0.08 (mean of triplicates).

4. 6. Small-scale Monoclonal Antibody Preparation and Purification

To avoid *in vivo* production of MAb in ascites fluid, lab scale production of P124 MAb in an *in vitro* system was initiated. Antibody secreting hybridoma P124.B2 was adapted to medium containing 5% FBS. $25\text{x}10^6$ cells were added to 250 mL of 5% FBS in DMEM and 1% OHCF. One week after inoculation, the supernatant was harvested. Cell viability did not fall under 25%. Thus, the cells were tested for growth in 1000 mL of 5% FBS in DMEM media.

In vitro production of LPS O157 antibodies was further carried out in SiCulture Bag ™. Approximately $50\text{x}10^6$ cells adjusted to grow in low percentage FBS, were diluted in DMEM (1:10) and injected into a bioreactor and the SiCulture Bag ™ was filled with 1800 mL of 10% FBS in DMEM. The viability of the cells was determined on a weekly basis and viability of the cells was 15% at the day of termination the biorection. At the beginning of the fourth week after the inoculation, the color of the nutrient medium changed from bright orange, pH= 7.2, to a yellowish salmon-pink indicating a slightly acidic pH. Approximately 1.75 L of supernatant was collected from the SiCulture Bag ™. The crude mixture of proteins from the one liter of the cell free supernatant was precipitated with 55% ammonium sulfate solution. The precipitate was resuspended in PBS and dialyzed against PBS. Approximately 50 mL (20 times volume reduction) was obtained after dialysis. The significance of this step is the reduction of volume from 1 L to 50 mL.

4. 6. 1. Purification and Characterization of P124 MAb

To purify MAb P124, an affinity chromatography column of protein G covalently attached to Sepharose was used. Since the method involves purification of IgG at different pHs and different buffer systems, the retained material was desorbed and examined along with unbound fractions by the ELISA method. The protein G affinity chromatography elution profile of the P124 MAb is shown in Figure 25 along with the details of the loading, wash, and elution conditions. Two peaks of antibody activity were seen indicating that unbound fractions also had considerable activity.

The amount of purified protein was determined by the Bradford method and spectrophotometric absorbance at 280 nm. The total amount of purified IgG recovered was 9.5 mg. The purity of protein P124 was further confirmed by SDS-PAGE electrophoresis, which indicated the presence of two major protein bands at 45 kDa and 21.5 kDa (Figure 26). The unbound fractions were reloaded on the protein G column to recover the P124 IgG.

4. 7. Cross-reactivity

To investigate the cross-reactivity, an assay of P124 MAb to several purified antigens was evaluated, using microtiter plates coated with these antigens. Various non-pathogenic strains of *E. coli* bacteria were grown on the agar plates at 37 ^{0}C for 15 h, and then inoculated in Luria-Bertani media at 37 ^{0}C for 15 h. Bacteria were purified by centrifugation with three PBS washings. Polystyrene plates were coated at antigen concentrations ranging from 10^5 -10^9 CFU/well in PBS. The cross-reactivity of P124 MAb was examined using purified *E. coli* JM 109, BL21 DE3 and Top 10'F strains of non-pathogenic bacteria. MAb P124 did not recognize the epitopes in non-*E. coli* O157 strains, as shown in Figure 27. The average absorbance for all tested clones producing MAb binding to non-*E. coli* O157 epitopes was less than 0.2.

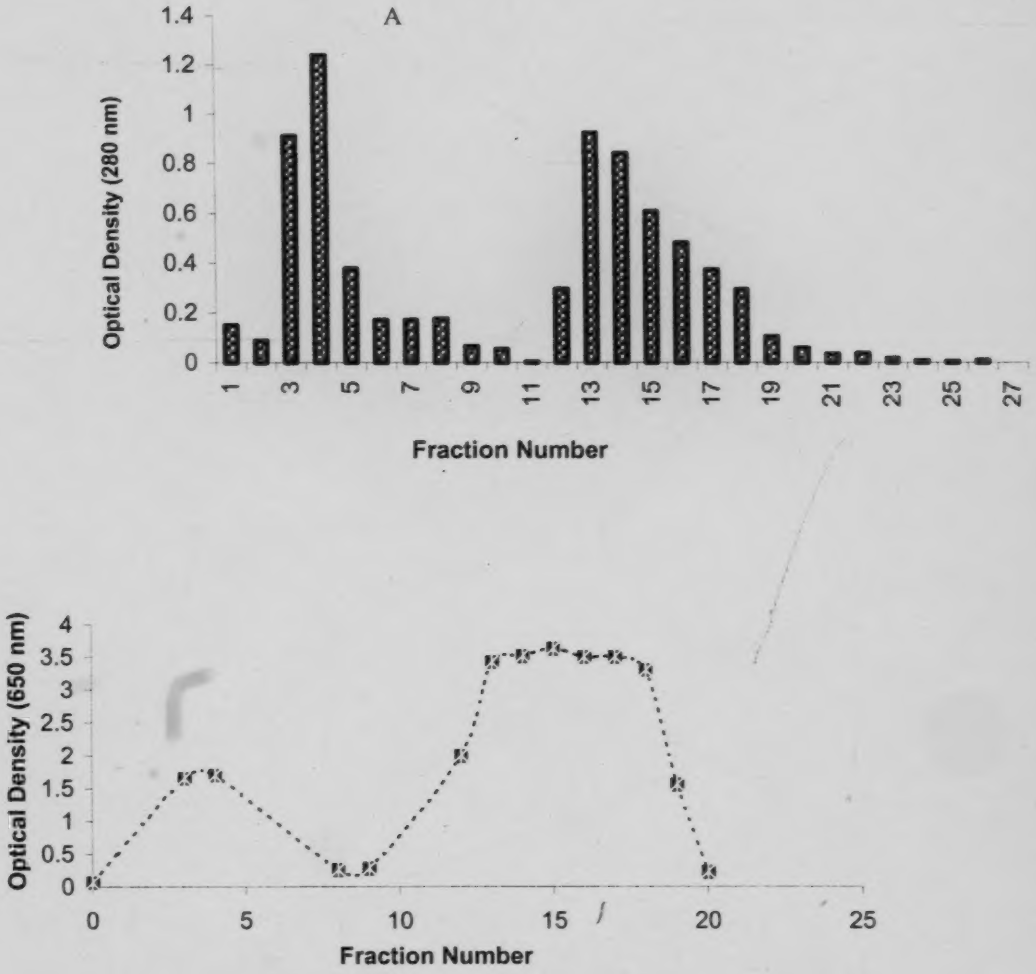

Figure 25: Protein G affinity chromatography of anti-LPS O157:H7 monoclonal antibody. Cell line P124.2A2-D10 was innoculated in a SiCulture Bag^{TM} antibody production device. The cell free supernatant was harvested at day 30 by centrifugation. The supernatant was precipitated by ammonium sulfate, dissolved in PBS and dialyzed against PBS, pH 7.2. The sample was then loaded on a Protein G column of 5 mL volume at a flow rate of 1 mL/min. After washing the column with 0.1 M sodium acetate buffer at pH 5.0, the bound antibodies were eluted with 0.1 M glycine hydrochloride,(pH 2.8) at a flow rate of 0.5 mL/min. The 1 mL fractions of unbound proteins (A, 1-10) and bound proteins (A, 11-20) were collected in a Tris buffer, (pH 7.5). Graph A shows the A_{280} absorbance data for each fraction. OD_{650} presented by the peroxidase activity after ELISA was performed in duplicates. Separation was good but not complete since there was about 30% immunoactivity in the unbound fraction. A second separation cycle was performed on the unbound fraction with Protein G affinity chromatography to recover additional MAb.

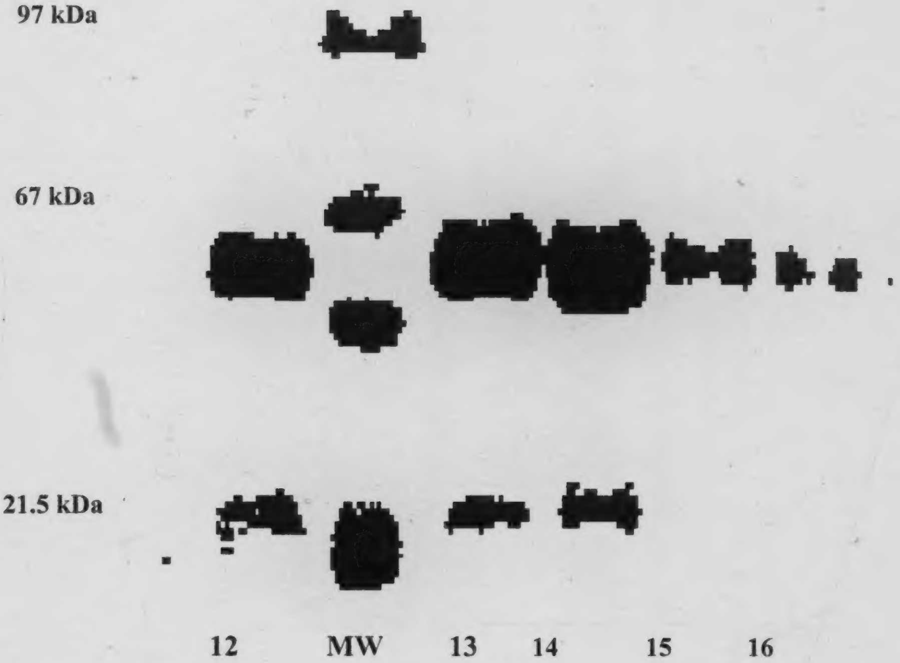

Figure 26: SDS-PAGE of Protein G column purified P124. The samples were electrophoresed on 12% SDS poly-acrylamide gel at 150 V for 1.5 h and the proteins were visualized by Coomasie blue staining. MW: Molecular mass standard (N. E. Biolabs Inc.). Lanes 12, 13, 14, 15, 16 are fractions 12, 13, 14, 15, and 16 from the elution peaks of the Protein G chromatography. Anti-LPS O157 MAb (P124) is seen in all fractions with the characteristic heavy and light chain around 45 kDa and 21.5 kDa bands.

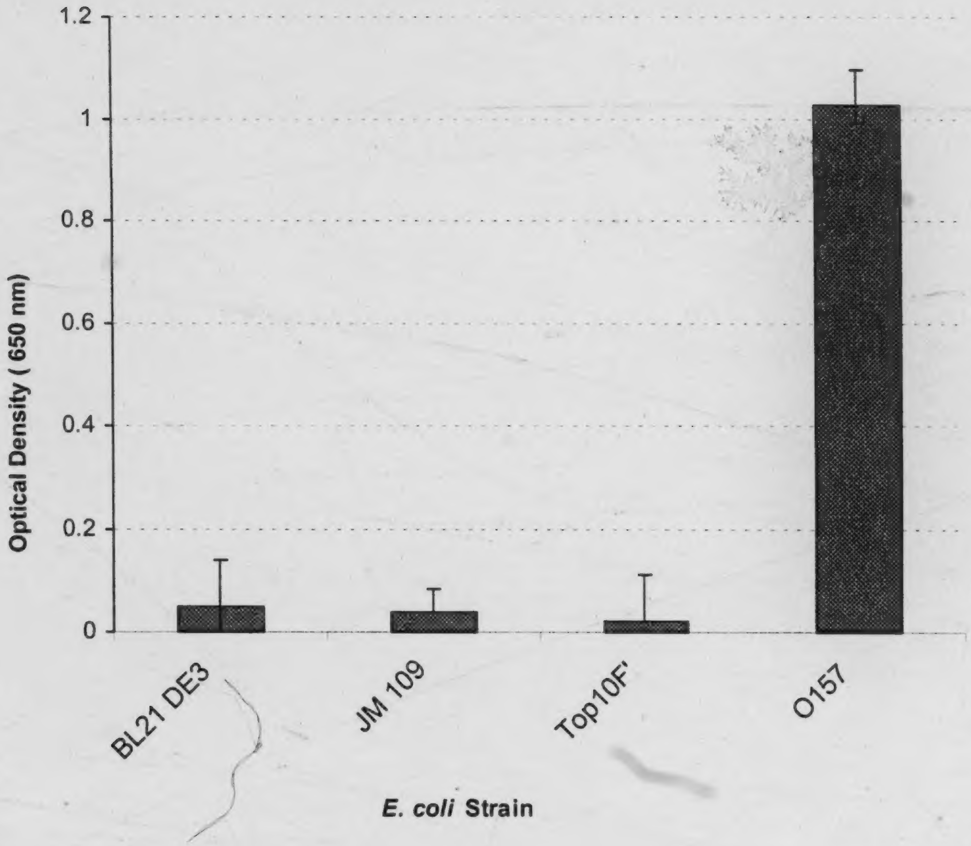

Figure 27: Cross-reactivity of anti-LPS O157:H7 monoclonal antibody with non-*E. coli* O157 strains. Cross-reactivity was determined by ELISA for eight cell lines producing P124 anti-LPS O157:H7 monoclonal antibodies. Absorbance readings at a fixed antibody dilution are shown for *E. coli* JM109, BL21 DE3, Top 10'F and *E. coli* O157:H7 (10^8 CFU/well of *E. coli* JM 109, BL 21 DE3, and Top 10F' strains). Diluted supernatants (1:20) were used in an indirect labeling assay using HRPO-goat anti-mouse IgG (1:10000) assay. Bars are standard deviations of duplicate studies for all eight clones grouped together with non-*E. coli* O157:H7 strains and *E. coli* O157:H7 heat killed bacteria. Phosphate buffered saline was used as a negative control to determine the background optical density (0.07).

On the other hand, *E. coli* O157:H7 reacted strongly with various dilutions of the P124 MAb. The average absorbance for the tested MAb O157 was greater than 1.0. The epitope in *E. coli* O157:H7 recognized by purified MAb P124 demonstrated good specificity and suggested that the MAb may be a good reagent for specific detection of the pathogen. However, further screening with clinical isolates is essential.

4. 8. Indirect Fluorescent-Antibody Assay for *E. coli* O157

The surface of *E.coli* O157:H7 bacteria was examined for its binding ability of purified monoclonal antibody P124 using an epifluorescence microscope and indirect immunofluorescence. The microscope chambers were coated with 1 mL of live bacteria (10^8 CFU) harvested from TSB growing media. The chambered slides were allowed to air dry before they were fixed in cold (-20 ^{0}C) acetone for 10 minutes. Two mL of P124 MAb (5 μg and 2 μg) was added to the slides. Dilution buffer was used as a negative control. After incubation at 37 ^{0}C for 2 h, the slides were washed extensively with ice-cold PBS-T-HS washing buffer. The non-specific binding sites were blocked with 2% DBSA-T. The reactive isothiocyanate form of fluorescein was conjugated to goat anti-mouse IgG at 4 °C and used as the indirect antibody to detect bound P124 MAb on *E. coli* O157:H7 bacteria. The slides were viewed on a Zeiss confocal microscope under excitation/emission at 488/530 nm with a 40-magnification objective (Figure 28). All dilutions of the MAb P124 exhibited binding to the bacterial surface and staining of these colonies consistently produced strong fluorescence. Nonspecific binding could be excluded since no labelling was seen when incubating conjugated mouse anti-immunoglobulin was incubated without the primary P124 MAb.

Figure 28: Detection of *E. coli* O157:H7 bacteria with anti-LPS O157:H7 monoclonal antibody by epifluorescence microscopy. 5 μg and 2 μg aliquots of P124 MAb in PBS were incubated with 10^8 CFU *E. coli* O157:H7 for 2 h at 37 ^{0}C. Fluorescein isothiocyanate conjugated to goat anti-mouse IgG (1:50) was incubated with the bound antibody for 30 minutes at 4 ^{0}C in a foil wrapped chamber. Then the slides were washed 6 times with cold PBS, fixed with 1% formaldehyde and mounted with 2.5% Dapco: 90 % glycerol in PBS. A 22 mm^2 cover glass was placed on the top of the slides. The slides were viewed with Zeiss LSM 510 confocal microscope under excitation/emission at 488/530 nm by using a 40-magnification objective. A given field reveals the *E. coli* bacteria binding with anti-LPS O157 monoclonal antibody.

The absence of fluorescence on the negative control slides suggests that the interaction between *E. coli* O157 epitopes and its MAb were specific and could provide the conceptual basis for a simple inexpensive assay to detect the pathogenic *E. coli* O157.

4. 9. Conventional, Fluorescence, and Chemiluminescence Homosandwich Immunossays

The previously developed ELISA was improved by replacing the polyclonal anti *E. coli* O157:H7 LPS serum with P124 MAb. Employing our new MAb P124, the homosandwich design was used to optimize the detection of whole *E. coli* O157 and the LPS. Further enhancement of sensitivity for the multivalent LPS epitopes was obtained by using a two-site homosandwich immunoassay. Colorimetric, fluorometric, and chemiluminescenct detection were evaluated for their relative sensitivities.

4. 9. 1. Design of Homosandwich ELISA

A high-binding 96-well microplate was used for the sandwich ELISA. One hundred μL of the primary P124 antibody was coated on the microplate directly by simply incubating the microplate with the antibody solution at 37 ^{0}C for 1 h. The preferred pH of the coating buffer was 9.6. The concentration of the coating antibody was 20 μg/mL. The microtiter plate was washed with excess PBS-T-HS to eliminate unbound antibodies. Diallyzed 2% BSA–0.001% Tween 20 in PBS was applied to the two-step homosandwich ELISA as a blocking agent (Figure 29). The aim of a low background in the homosandwich ELISA was achieved by repeated washing with buffer for 6 cycles. The mean OD_{650} for the negative control did not exceed a value of 0.11.

The design of the two-step homosandwich assay included incubating heat killed *E. coli* O157 or LPS preparations (as micelles in aqueous buffers) to be captured by the antibody coated solid phase. Following the wash step, the biotin-labeled P124 is added, washed, and streptavidin-HRPO is then added. After the final wash, the various substrates were added for signal generation.

4. 9. 2. Assay Optimization

4. 9. 2. 1. Non-specific Binding

Initially, the ELISA assay backgrounds were high and nonspecific binding of some components was suspected. Optimization of blocking non-specific binding was tested using fish gelatin, dry non-fat milk, undialyzed BSA, dialyzed BSA, and FBS (Figure 29). The signal-to-noise ratio, the most important parameter when selecting a blocking agent, was measured as the signal obtained with a sample containing the O157 antibody compared to the signal obtained without the antibody. Non-fat dry milk at 0.5% concentration showed the most well-to-well inconsistency and the highest background noise. The following reagents reduced non-specific background in the following order: 2% BSA> 0.25% fish gelatin or 2% DBSA> 5% DBSA> 2% FBS> 10% FBS. Further evaluation of blocking agents showed that the most cost effective

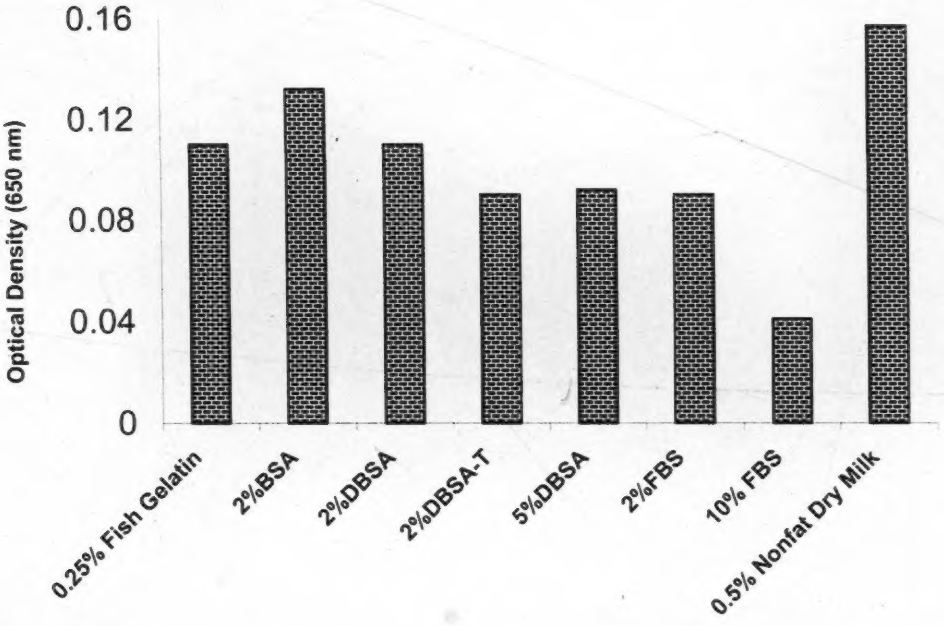

Figure 29: Optimization of blocking to reduce non-specific binding. In order to avoid high background noise, several blocking buffers were evaluated. This standard immuno assay was performed on polystyrene 96 microwells coated with 10^8 CFU/well *E. coli* O157:H7 heat killed bacteria and incubated for 2 h at 37 ^{0}C. The unreactive sites on the polystyrene surface and nonspecific binding proteins were blocked with 250 L of: 0.25% fish gelatin (first left bar), 2% non-dialyzed bovine serum albumin (BSA), dialyzed bovine serum albumin (DBSA) (2% and 5%), 2% dialyzed bovine serum albumin- 0.001% Tween 20 (DBSA-T), fetal bovine serum (FBS) (2% and 10%), and 0.5% nonfat dry milk in PBS, pH 7.2. The plates were blocked for 1 h at 37 ^{0}C followed by 15 h at 4 ^{0}C. The plates were washed with PBS-T buffer. Two g of 13B3 MAb (external positive O157MAb control) in dilution buffer or 100 L serum antibody (internal positive control-serum from mouse immunized with *E. coli* O157) (1:20 dilution) was added in replicates of eight. The remaining wells were incubated with 100 L PBS-0.001% Tween 20-0.1% BSA at pH 7.4 for 1.5 h at 37 ^{0}C. The signal generated by the TMB substrate was measured. Optical density of positive control was 0.7 and 1.2, respectively

blocking was obtained with 250 μL of 2% purified BSA in PBS after 1 h at 37 ^{0}C and 15 h at 4 ^{0}C. The non-ionic detergent, 0.001% Polyoxyethylene 20 sorbitan monolaurate (Tween 20), was added to the 2% purified BSA The dialyzed BSA-Tween blocking agent stabilizes the coated antibody and reduces non-specific adsorption onto the walls of the plates. These reagents lead to improved precision of the immunoassay as shown by increased signal-to-noise ratios.

4. 9. 2. 2. Variations of Incubation and Temperature

The antibody-antigen reaction is temperature dependent. Since the association of antigen and antibody occurs between about 0 to 40 ^{0}C. The reactants wer incubated at RT and 37 ^{0}C. The effect of incubation time was investigated by incubating the *E. coli* O157 bacteria with anti-LPS O157 for 0.5, 2, 3, and 15 h at both room temperature and 37 ^{0}C. Prolonging the incubation time over 2 h did not significantly increase the signal at 37 ^{0}C. On the other hand, incubation of *E. coli* O157:H7 with anti-LPS O157:H7 antibody for 15 h at 37 ^{0}C resulted in increased background with OD_{650} higher than 0.2.

Based on the data shown in Figure 30, 2 h incubation time at 37 ^{0}C and 30 min of stabilization in color development was found to be ideal.

4. 9. 2. 3. Concentration of Coated Antigen

A decrease in ELISA readings was seen at increasing concentrations of the antigen in the coating buffer (Figure 31). The results in Figure 31 show that 10^7 – 10^8 CFU/well is adequate for optimal signal.

In addition, the optimization of all steps of the anti-LPS O157:H7 test was carried out in designing the enzyme immunoassay so that the

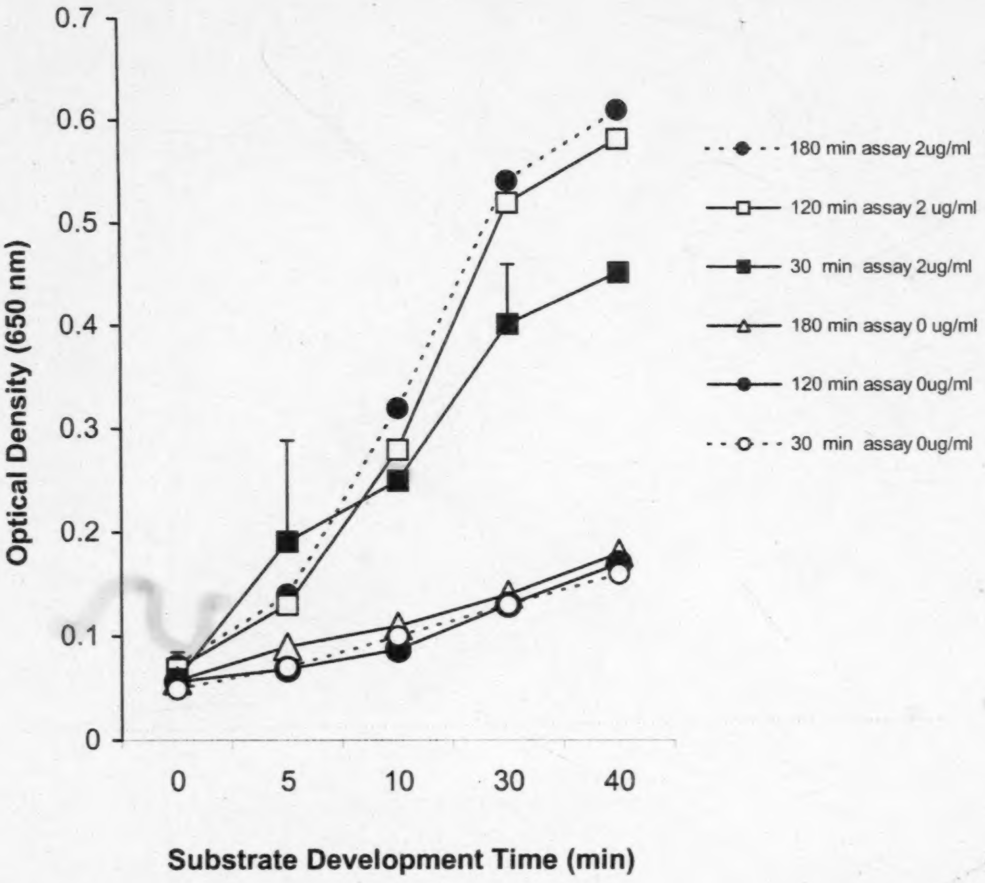

Figure 30: Time effect on TMB/H_2O_2 color development for assay signal. To evaluate the time path of TMB signal development and the effect of incubation time of anti-LPS O157:H7 monoclonal antibody binding to antigen, a direct ELISA of 2 μg/mL P124 in triplicate was performed. Three time plots of 30 minutes, 2 h, and 3 h incubations of P124 with 10^8 *E. coli* O157:H7 whole cells at 37 ^{0}C were studied. The enzyme labeled second antibody goat anti-mouse IgG conjugated to HRPO (1:10 000 dilution) in PBS-T was added to each well and incubated for 1 h at 37 ^{0}C. To demonstrate the importance of time in signal development by TMB substrate, the optical density was recorded at 650 nm at 5 min, 10 min, 30 min, and 40 min of reaction.

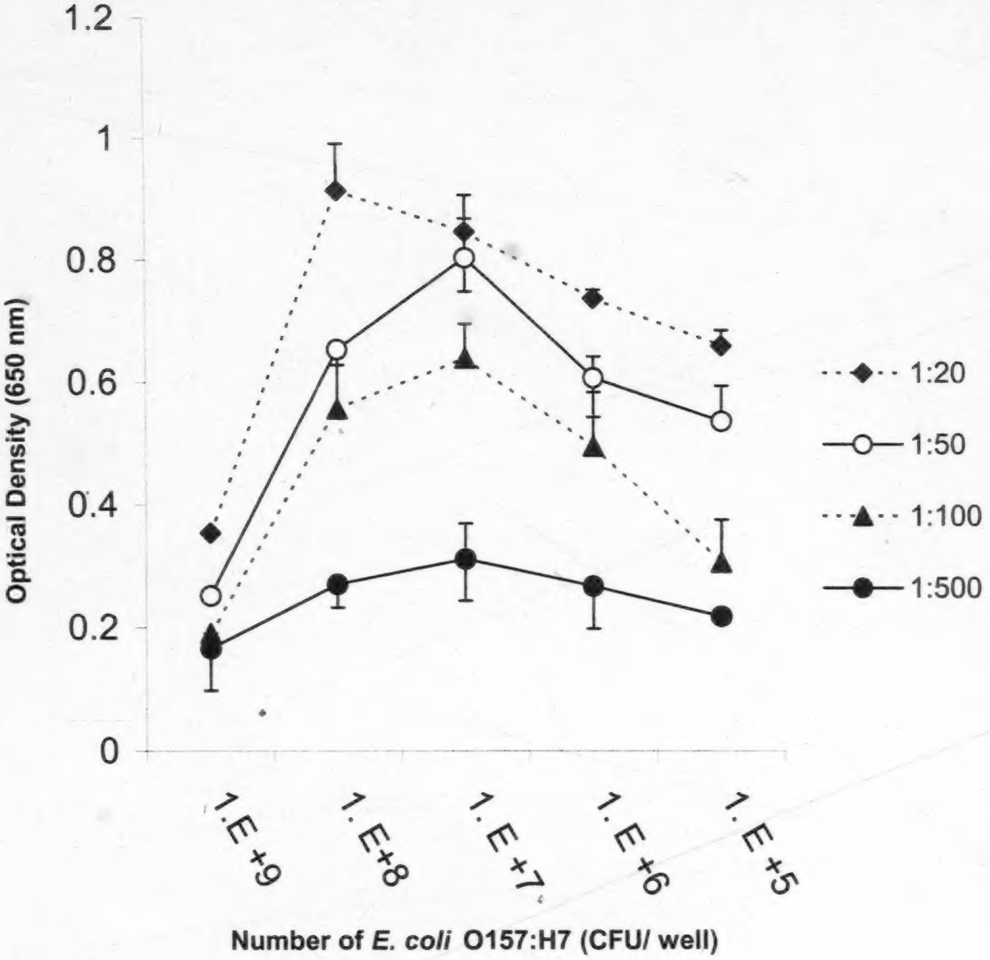

Figure 31: The effect of concentration of *E. coli* O157:H7 antigen on the ELISA value. The optimal concentration of *E. coli* O157:H7 (10^5,10^6, 10^7, 10^8, and 10^9 CFU/well) for hybridoma screening was estimated by performing an ELISA assay. Standard antibody was added to each 96-well plate in duplicates at dilution ratios of 1:20, 1: 50, 1:100, and 1:500 in 0.05 M bicarbonate buffer, pH 9.6. The assay was developed with TMB and sample absorbance at 650 nm was measured.

OD_{650} was in range of about 0.1 to 1.5. Based on the limitations of UV/VIS spectroscopy, the optical density of the chromophore substrate TMB/H_2O_2 was kept under 1.5. This ensures a linear correlation between the absorbance and the concentration of the analyte.

4. 9. 3. Colorimetric, Fluorometric, and Chemiluminescence Detection

Conventional colorimetric, chemiluminescence, and fluorometric detection were evaluated with various concentrations (4-0.004 µg/well) of biotinylated P124 monoclonal antibody using a sandwich two-step ELISA for binding to various concentrations (20-0.002 µg/mL) of LPS O157 antigen. Streptavidin–HRPO (1:10 000) bridged the antibody-antigen homosandwich cluster and were incubated with the corresponding substrate (TMB for colorimetric, QuantaBlu™ for fluorometric, Pico™ or Femto™ for chemiluminescent detection). Approximately 0.4 µg/well of tracer antibody was sufficient to bind all the various concentrations of the multivalent LPS O157 antigen.

The relative sensitivities of the conventional TMB immunoassay (Figure 32), the fluorometric QuantaBlu™ immunoassay (Figure 33), and the chemiluminescent Pico™ and Femto™ immunoassays (Figure 34 and Figure 35) revealed a chemiluminescence signal which increased over more than three orders of magnitude in LPS concentration and had the best sensitivity for O157 LPS detection (Figure 36).

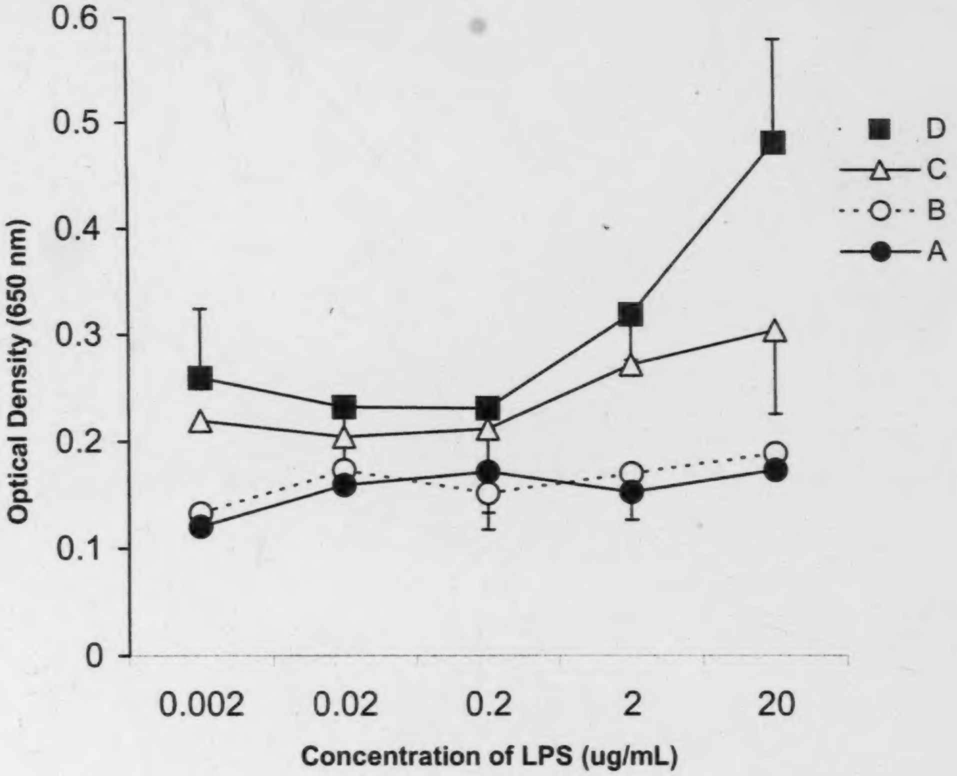

Figure 32: Determination of the optimal concentration of tracer anti-LPS O157 antibodies for a conventional two-site homosandwich immunoassay. The optimal concentration of the biotin labelled P124 monoclonal antibody was evaluated for detection of various concentrations of LPS O157:H7 by conventional ELISA. The signal generated from 2 μg/well purified P124 capture antibody, blocked with 2% DBSA-0.001%Tween, and incubated with 20 μg/well, 2 μg/well, 0.2 μg/well, 0.02 μg/well, and 0.02 μg/well of LPS O157 in triplicate were compared with streptavidin-HRPO bonded to tracer biotinylated P124 antibody in A= 0.004 μg/well, B= 0.04 μg/well, C= 0.4 μg/well, and D= 4 μg/well. The TMB colorimetric substrate signal measured at 650 nm optical density was used.

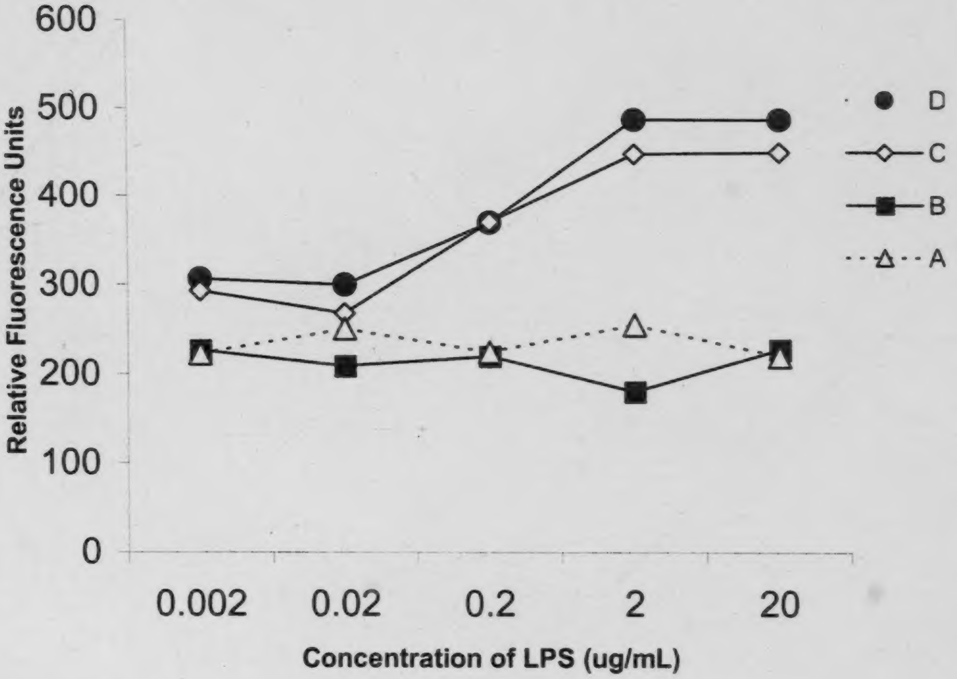

Figure 33: Fluorescence homosandwich ELISA utilizing QuantaBlu™ fluorogenic substrate. Optimization of biotinylated tracer P124 monoclonal antibody for recognizing specific multivalent LPS O157 antigen in various concentrations were evaluated by fluorescence of QuantaBlu™ in homosandwich ELISA. The sensitivity of detection as a function of both coating antibody and biotinylated concentrate (A= 0.004 µg/well, B= 0.04 µg/well, C= 0.4 µg/well, and D= 4 µg/well) were compared. The relatively low signal-to-noise response can be related to the high background noise created by the initial absorption of light at 546 nm followed by emission at 575 nm. Background due to binding was eliminated, as shown in previous tests, by multiple washing of the plates.

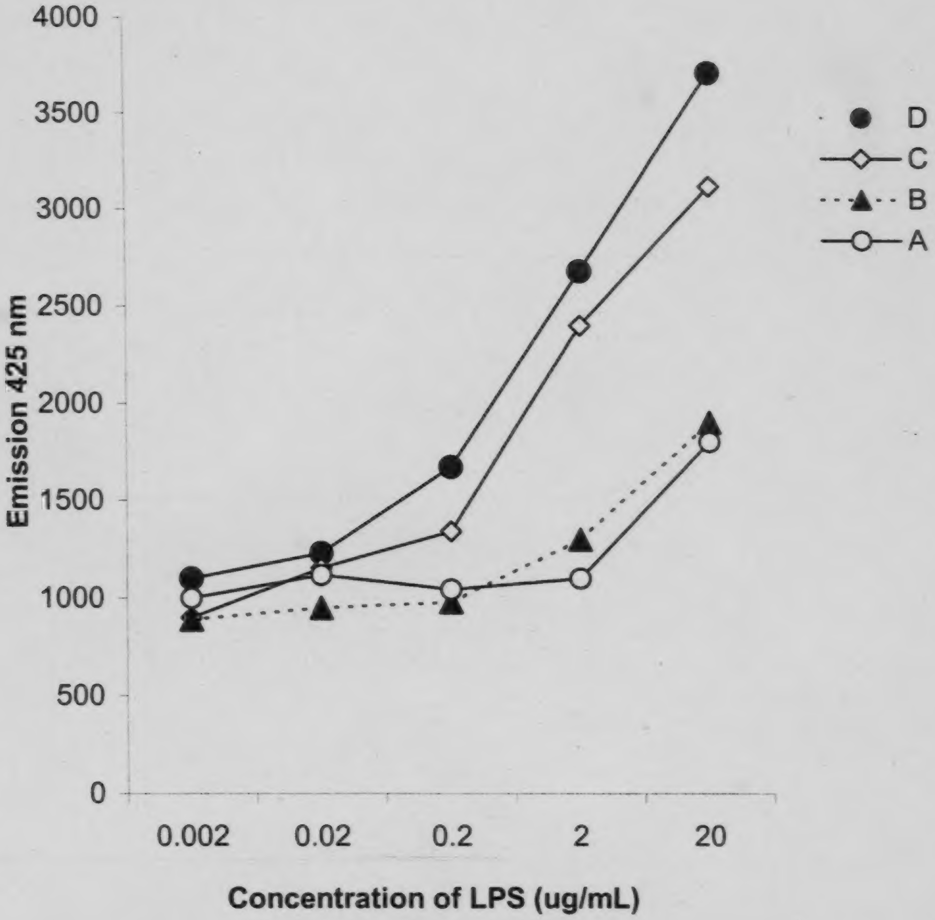

Figure 34: Chemiluminescence homosandwich ELISA utilizing Pico™ chemiluminescent substrate. Optimization of biotinylated tracer P124 monoclonal antibody for recognizing specific multivalent LPS O157 antigen in various concentrations were evaluated by activation of chemiluminescent Pico™ substrate. A 96-well microplate was coated with 2 μg/well of purified P124 anti-LPS monoclonal antibody and blocked with 2% DBSA-T. 20 μg/well, 2 μg/well, 0.2 μg/well, 0.02 μg/well , and 0.002 μg/well of LPS O157 in duplicates were incubated for 2 h at 37 °C. Unbound antigens are removed by multiple washings with cold PBS-T-HS, pH 7.2 and the detecting antibody to bind to the antigen. Detecting biotinylated P124 antibody was added as A= 0.004 μg/well, B= 0.04 μg/well, C= 0.4, and D= 4 μg/well and incubated for 1 h at 37 °C. Strptavidin conjugated horseradish peroxidase (1:10 000) reporter was added for 1 h incubation at 37 °C. Subsequently 100 μ L of Pico™: H_2O_2 substrate was added and developed.

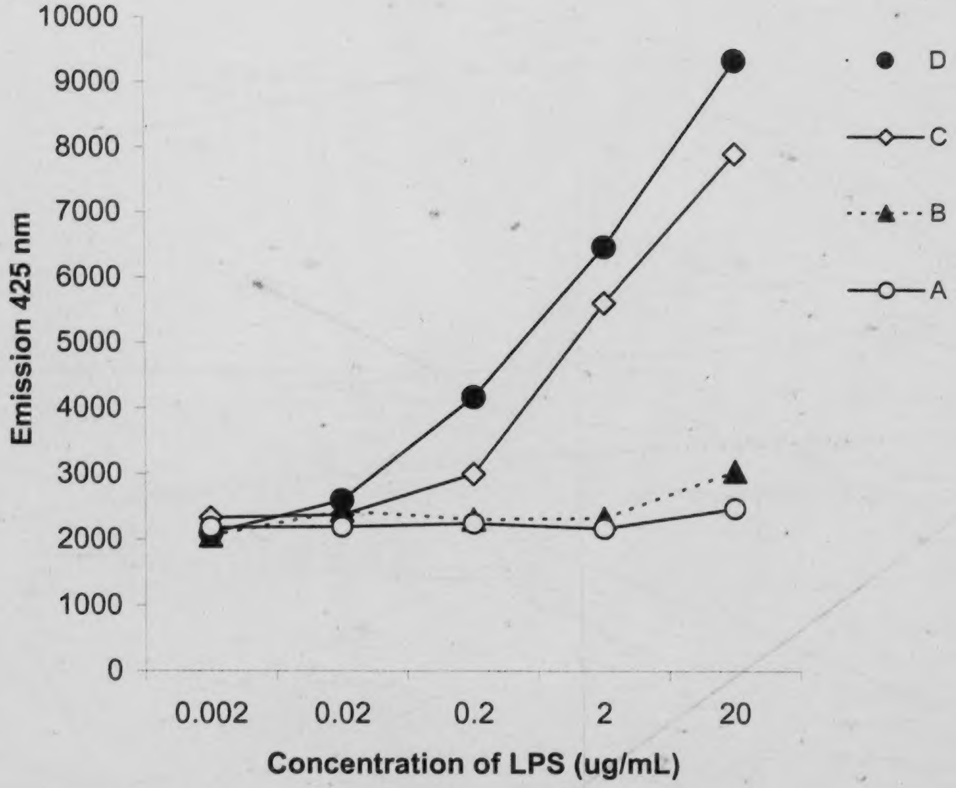

Figure 35: Chemiluminescence homosandwich ELISA utilizing Femto^{TM} chemiluminescent substrate. Optimization of biotinylated tracer P124 monoclonal antibody for recognizing specific multivalent LPS O157 antigen in various concentrations was evaluated by activation of chemiluminescent Femto^{TM} substrate by the peroxidase-conjugated signal antibody. The concentration of product from the enzymatic reaction of bound signal antibody is directly proportional to the concentration of the tested antigen. Varying amounts of biotinylated P124 MAb conjugates (A= 0.004 µg/well, B= 0.04 µg/well, C= 0.4 µg/well, and D= 4 µg/well were added, washed, and HRPO-streptavidin was added. Following a final wash, the Femto^{TM} substratewas added and signal measured. Mean of background activity was 633. The points represent mean of duplicates.

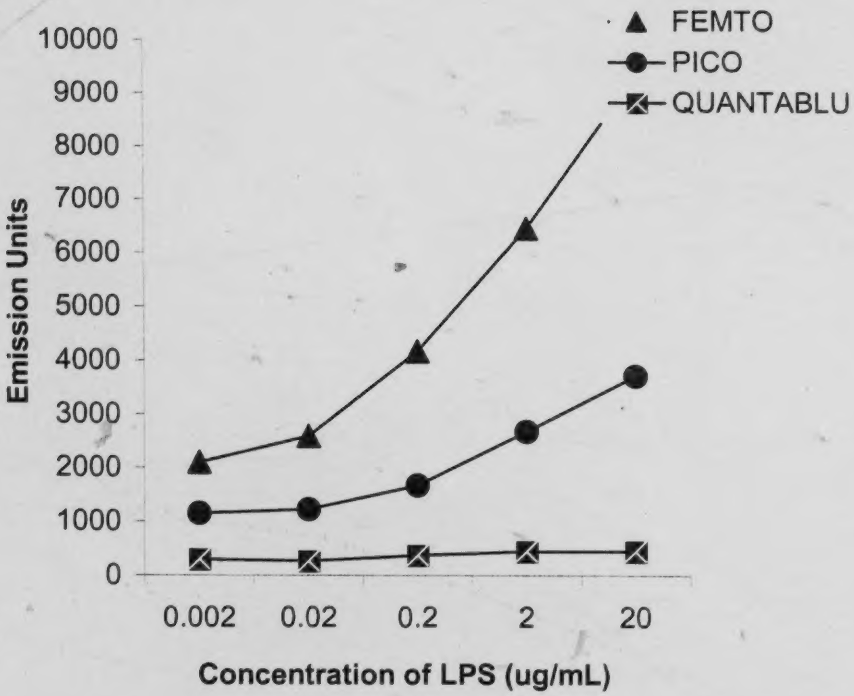

Figure 36: Comparative evaluations of fluorometric and chemiluminescent substrates. This is a relative measure within 25 duplicates of negative control with no antigen and sextuplicates of various levels of LPS. The signal generated from 2 µg/well purified P124 capture antibody, blocked with 2%DBSA-0.001%Tween, and incubated with 20 µg/well, 2 µg/well, 0.2 µg/well, 0.02 µg/well, and 0.02 µg/well of LPS O157 were compared with streptavidin-HRPO bonded to 0.4 µg/well tracer biotinylated P124 antibody. The fluorescence QuantaBlu^{TM} substrate signal was measured by emission at 575 nm, and activation of chemiluminescent Pico^{TM} and Femto^{TM} substrate was measured by emission at 425 nm.

Detection Method	**Peroxidase Substrate**	**Lower Limit of LPS O157 Detection**
Colorimetric	TMB	2 μg/mL
Fluorometric	QuantaBlu^{TM}	0.2 μg/mL
Chemiluminescent	Pico^{TM}	0.2 μg/mL
Chemiluminescent	Femto^{TM}	0.02 μg/mL

Table 9: Sensitivities of LPS detection by various substrates. ELISA homosandwich system was designed for various amount of LPS analyte (0.002 μg/mL- 20 μg/ml). Analyte was estimated by binding 4 different concentrations of tracer antibody (0.004μg/well – 4μg/well). The limited study results presented here suggests that Femto^{TM} appears the most sensitive assay. Additional studies need to be done to validate the above results with LPS obtained from various strains of *E. coli* O157.

5. DISCUSSION

The initial objective was to develop a monoclonal antibody against the carbohydrate epitope of the *E. coli* O157:H7 LPS for specific diagnosis of this human pathogen. Recent detection methods focus on the whole cells of *E. coli* O157:H7 rather than the LPS O157:H7 fraction (Johnson et al., 1995; Meng et al., 1996; Feldisine et al., 1997; Fratamico et al., 1997). Most commercially available enzyme-linked immunosorbent assays incorporate a polyclonal *E. coli* O157:H7 antibody instead of a monoclonal antibody (Park et al., 1994; Okrend et al., 1992; Bennett et al., 1996; Chapman et al., 1997; Tsai et al., 2000). There might be two reasons for this: the relative ease and low cost of preparation of the polyclonal antibody and the limited commercial availability of monoclonal antibody to *E. coli* O157:H7.

The cross-reactivity and immunoassay experiments showed that the anti-O157 P124 monoclonal antibodies developed exhibited good specificity and sensitivity (Figure 27). The anti-LPS O157:H7 monoclonal antibody produced in this study, and the anti-*E. coli* O157 monoclonal antibodies prepared previously (Perry et al., 1988; Padhye et al., 1991; Helander et al., 1997; and Westerman et al., 1997) are highly specific diagnostic reagents. In this study the monoclonal antibody P124 was tested to identify *E. coli* O157:H7 heat killed whole cells as well as extracted LPS O157:H7 by colorimetric, fluorometric, and chemiluminescence immunoassays (Figures 32-35). The labelled P124 monoclonal antibody samples were also evaluated with *E. coli* O157:H7 bacteria by epifluorescence microscopy (Figure 28). In addition, it has also been shown from these studies that aggressive immunization to stimulate the humoral immune system and close monitoring of the IgG activity in serum are important for specific hybridoma production (Table 2, Figure 22, and Table 8).

Preparation of the antigen

The first goal was to prepare a sufficient amount of antigen for immunization and for the screening of potential anti-LPS O157:H7 hybridoma clones. There have been several techniques described to isolate LPS. One extracts the smooth and rough LPS using acetone (Proux *et al.*, 2002). However, the majority of researchers extract LPS with the phenol-water procedure (Westphal, and Jann, 1965). Modified versions of the phenol-water LPS extraction have been utilized with several microorganisms; for example, *Rhizobium fredii, Vibrio vulnificus, Actinobacillus actinomycetemcomitans, A. pleuropneumoniae, Escherichia coli* O21, *Citrobacter freundii, E. coli* O157:H7; (Yang *et al.*, 1998; Reddy *et al.*, 1998; Blix *et al.* 1999; Staaf *et al.*, 1999; Chart *et al.*, 1993; Perry *et al.*, 1986; Westerman *et al.*, 1997; Nishiuchi *et al.*, 2000). Section 3. 2. of this thesis describes the methods used for the extract LPS in the development and characterization of the P124 monoclonal antibody.

Smooth strains of bacteria (S-LPS) can survive in the host by the ability to express a mixture of protective O-LPS with different chain lengths and unsubstituted complete core LPS (R-LPS). On the other hand, rough-mutant bacteria (R-LPS) are serum-sensitive and are lysed the presence of complement. It has been observed that the length of O-chain is critical for their extractability (Chart *et al.*, 1993). The LPS of *E. coli* O157 is hydrophobic and lipophilic; thus the phenol phase of the hot phenol-water method extracts the long chain O-LPS (Perry *et al.* 1986, Westerman *et al.*, 1997). The data present (Figure 17, and Figure 18) supports the previous findings. It was somewhat unexpected to see that anti-LPS antibody reacted with the antigen, which was soluble in the water phase. One possibility of the water-soluble LPS is alterations caused to the cell surface by the removal of LPS. It has been reported that hydrophilic short chain LPS *Citrobacter freundii* could be extracted into the aqueous phase (Chart *et al.*, 1993; Nishiuchi *et al.*, 2000). Similarly, core-LPS O157 or fragments of long chain LPS derived from *E. coli* O157:H7 could be extracted in the water phase. The water phase LPS O157 extract was evaluated for reactions with anti-LPS O157 antibody. The findings presented in Figure 17 and Figure 18 add to growing evidence that the water phase extract contains a mixture of rough-LPS and detached pieces of long chain LPS. Selective screening of primary clones and subclones was directed towards the LPS O157:H7 phenol extract in order to isolate the specific antibody to LPS O157:H7 (Table 5 and Table 8).

Detection and measurement of endotoxin

LPS, the endotoxic outer membrane component of gram-negative bacteria, has long been implicated as an indicator in the pathophysiology of septic shock. The rabbit pyrogen test was developed in the 1940's as the first method for detecting endotoxin contamination. Recently, alternative endotoxin testing is provided by the monocytic cell line Mono Mac 6 assay (Moesby, 2000). In 1956, Bang noted that amoebocytes, blood cells from the horseshoe crab, could bind to the outer cell wall structures of microbial cells, releasing the clotting factor coagulen, causing a blood clotting reaction.

Since the 1970's, a semi-quantitative clotting assay has been utilized in most determinations of LPS (Watson and Hobbie, 1977). In 1980, the *Limulus* Amoebocyte Lysate (LAL)-assay was adapted as the standard assay for endotoxin detection by the American Food and Drug Administration (FDA). There are four commercially available tests currently approved by FDA, namely: gel-clot, turbidimetric, colorimetric, and the chromogenic assays (Associates of Cape Cod Incorporated bulletin, 2002). A generally accepted protocol is not yet available (Heederik, 1997) due to the pros and cons of each method.

Instead of using a semi-quantitative estimation of endotoxin, the specific amount of endotoxin in isolated LPS was determined by the more recent chromogenic version of the LAL-assay. The LAL assay is very sensitive with a range of 0.01-100 Endotoxin Units (EU)/mL. A standard endotoxin curve was developed with the control standard endotoxin (*E. coli* O113:H10) of known concentration. In addition to CSE (*E. coli* O113:H10) activity, Internal Control Standard Endotoxin (ISCE) was estimated in the samples of known concentrations for LPS O157 (kindly provided by Dr. Conlan, NRC, Ottawa) and *Salmonella* LPS O30 (Dr. Bundle, Dept of Chemistry, U of A). The biological activity of the LPS O157 extracted by the hot-phenol method as described in this thesis is comparable to those of ICSE in terms of activation of the *Limulus* amoebocyte lysate enzyme (Figure 20).

Time course of antibody response

The approach to induce serum IgG anti-lipopolysaccharide was evaluated by immunizing mice with either a short-term immunization protocol often applied in our laboratory or by developing a long-term immunization protocol. Initially a combination of heat killed whole cells was administered in both protocols and then purified LPS prior to fusion was employed in a long-term immunization method. The goal of immunizations was to study the dose related specific antibody response (Table 3, and Figure 21). The immune response of mice to *E. coli* O157:H7 was studied by measuring the IgG and IgM serum antibody activity with specific secondary reagents. The mice were bled and serum IgG anti-LPS and IgM anti-LPS were measured by *E. coli* O157 heat killed whole cells used as a solid phase antigen (Figure 21). Group I mice treated with a low concentration of LPS over a short-time exposure (15-day protocol) elicited low amounts of antibody in the serum (Table 3). In a previous immunization protocol (Perry *et al.*, 1988), a one-week rest between the first two immunizations, followed by a 3-week rest and two final immunizations with 10-day rest in between injections was followed. A more elaborate regime was applied in an immunization protocol that lasted for 28 weeks (Helander *et al.*, 1997). It was shown that monoclonal antibodies were selected from four fusions resulting in 130 hybridomas. Another approach by Westerman *et al.* (1997) showed that an interval of 3 weeks between the first two immunizations and subsequent injections every two weeks (four to six) lead to the production of hybridoma, after several fusions. As shown in Table 2 and Figure 21, monitoring the development of the antibody response in the sera of the mice for the multivalent LPS antigen during the immunization protocol resulted in the selection of the best responders for hybridoma fusions. It was observed that while the mice had adequate serological response to the antigen (Figure 23, and Table 3), some fused spleen cells did not result in satisfactory antibody production. These results are consistent with earlier findings by other researchers (Helander *et al.*, 1997, and Westerman *et al.*, 1997).

Simultaneously with the time course of the immune response, the dose response was also studied for *E. coli* O157:H7 and purified LPS O157:H7. It is known that surface polysaccharides are critical virulence factors and a protective antigen for many Gram-negative bacterial pathogens (Robbins, 2001). Epitopes of polysaccharides are repeated in about 2 million copies on the outer membrane of *E. coli* bacteria. It is also known that the molecular weight of the polysaccharides is directly related to their ability to induce an immune response. Exhaustive testing failed to induce serum antibodies by isolated O-specific polysaccharides, considered as haptens (Conlan *et al.*, 2001). Several approaches to immunize mice for production of antibodies in spleen cells have been studied. Westerman *et al.* (1997) initially administered 10 μg of *E. coli* O157:H7 whole cells and 50 μg of crude flagella antigen per injection. Perry *et al.* (1988) immunized mice with 0.5 mL of 10^8 *E. coli* O157:H7 phenol killed cells. Limited previous information could be found about the administration of LPS O157:H7 for immunization of mice. Although one report indicated that mice were injected intraperitoneally with as high as 25 μg of LPS (Zaks-Zilberman *et al.*, 1998). It has been shown that subpopulation of a high affinity antibody forming cells can be stimulated more efficiently with lower doses of antigen during the initial stages of the immune response (Werblin *et al.*, 1973).

In search for the most suitable dose of glycolipid-based antigen, it was found that previous exposure of B-cells with glycoproteins that contain cross-reactive oligosaccharides followed by stimulation with bacterial lipopolysaccharides elicited a high titer of antibodies to lipopolysaccharides or oligosaccharides (Anderson *et al.*, 1993). The antibody activity of IgG against the whole cell was higher for group II than for group I mice (Table 3). However, when LPS was used as an antigen, group III mice showed similar or even higher IgG activity after a combination of *E. coli* and LPS immunization. As shown in Table 3, lipopolysaccharide appeared to be the predominant immunogen and considerably stimulated IgG antibody response in conjunction with initial priming (Figure 22). However, no LPS stimulation was required to obtain a substantial IgM response (Figure 22). Injecting the antigen as a cocktail of LPS O157 and whole cells seemed to be more efficient in producing an immune response than giving the antigen in only one form of LPS. Furthermore, most of the antibodies produced exhibit good binding to the LPS O157 antigen (Table 5). The results indicate that priming the cells can improve the IgG response when the lipopolysaccharide is injected as an antigen. The serum anti-LPS IgG antibodies elicited by i.p. injection were a good indicator for the selection of the most suitable mouse for splenic cell fusion in the production of hybridoma (Figure 22).

ELISA screening of primary hybridomas

Screening the large number of clones for the most desirable clone, lead to the need to design an ELISA test with low background. Careful and rigorous washing lowered the nonspecific binding. Lindhorst *et al.* (1998) observed that molecules containing many carbohydrates clustered together, bind far more strongly to proteins than other molecules, which do not possess this multivalency. Further studies of multivalent sugar ligands in effort to understand carbohydrate-protein interactions revealed that the multiantennary carbohydrates, also called also octopus glycosides, bind far more strongly to proteins than expected (Lindhorst *et al.*, 2002). It was observed that multiple washing did not affect the detecting signal.

Carbohydrate structure and cross-reactivity

The cell-surface polysaccharide of *E. coli* O157 has received increasing attention in recent years. The simple and yet unusual chemical structure of these macromolecular carbohydrates has presented problems for detailed structural studies. However, through improved carbohydrate chromatography, NMR, and mass spectrometry techniques studies of epitopes have been made more practical. Recent reports on *Citrobacter freundii* OCU 158 indicated that the cross-reactive epitope was found to be identical by chemical and nuclear magnetic resonance analyses to an unbranched linear polymer of a tetrasaccharide repeating unit of 2-acetamido-2-deoxy-D-galactose-4-acetamido-4,6-

dideoxy-D-mannose-L-fucose, D-glucose as [-3)-α-D-GalNAcp-(1-2)-α-D-PerNAcp-(1-3)-α-L-Fucp-(1-4)-β-D-Glcp-(1-]$_n$ (Perry *et al.*, 1986; Nishiuchi *et al.*, 2000; Nishiuchi et al., 2002).The structure previously proposed as [-4)-β-D-Glc-(1-3)-α-D-PerNAC-(1-4)- α-D-GalNAc-(1-4)- α-D-GalNAc-(1-3)- α-L-Fuc-(1-]$_n$ by Nishiuchi (Nishiuchi *et al.*, 2000) was revised to the original linkage position of an unbreached linear polysaccharide proposed by Perry *et al.* in 1986.

E. coli O157 is perhaps one of the most dangerous enteric pathogen that researchers and clinical microbiologists are likely to encounter. The risk of laboratory-acquired infection with *E. coli* O157 would be eliminated by conducting experiments with equivalent non-hazardous research material. Since the structure of the O-specific polysaccharide from non-pathogenic *Citrobacter freundii* and *E. coli* O157:H7 pathogen share common epitopes, utilizing the safe *Citrobacter freundii* in the test for clinical or epidemiological studies has been proposed (Nishiuchi *et al.*, 2000).

Several other species of bacteria have been reported to show a cross-reactivity pattern similar to *E. coli* O157:H7. A previous extensive cross-reaction study of *E. coli* revealed that the LPS sugar 4-amino-4, 6, -dideoxy-α-D-mannopyranoside is a determinant in antibody cross-reactions between *E. coli* O157 and other bacteria. *Brucella abbortus* (Chart *et al.*, 1992; Stuart et al., 1982), *Brucella melitensis* (Schoerner, 1991), *Yersinia enterocolitica* O9 (Chart *et al.*, 1992; Bundle *et al.*, 1984) and certain strains of *Escherichia hermanii* (Rice *et al.*, 1992) were also shown to share epitopes on the LPS. Furthermore, the non-pathogenic strains *Citrobacter sedlakki* (Park *et al.*, 1998; Vinogradov et al., 2000), O:30 N *Salmonella* group and *Vibrio cholerae* O1 (Chart *et al.*, 1993) exhibit cross-reactivity with *E. coli* O157:H7. A prospective study of cross-reactivity was explored utilizing *Vibrio cholerae* in the development of an oral vaccine against *E. coli* O157:H7 pathogen.

A limited experiments in specificity of P124 against distinctive epitopes of the *E. coli* O157:H7 LPS was conducted from experiments with other non-pathogenic variant strains of *E. coli* bacterium. An estimation of P124 monoclonal antibody cross-reactivity was determined by incubation of a fixed antibody concentration with varying concentrations of non-*E.coli* O157:H7 strains. We tested the *E. coli* strains JM109, BL21 DE3, Top 10'F grown in L-B media, and *E. coli* O157:H7 grown in TSB media. Polystyrene plates were coated with the antigen at concentrations ranging from 10^4 -10^9 CFU/well. Analysis of the various *E. coli* strains showed that the purified monoclonal antibody was immunoreactive only with *E. coli* O157:H7 (Figure 27). The immunoreactivity of other *E. coli* group was nearly equal and to background levels. This study on the specificity and cross-reactivity of P124 MAb included only three commonly available laboratory strains. It is essential to further test several other clinical strains to confirm the diagnostic utility of P124.

Two-site sandwich assay

The principle of immunoassay is based on the specificity of the antibody to recognize and bind a particular antigen moiety and elicit a signal that qualitatively and quantitatively estimates the presence of the analyte (Ishikawa *et al.*, 1998). Two-site non-competitive sandwich immunoassays (Figure 16) rely on two antibodies with a solid phase antibody as the capture antibody and a second antibody conjugated to an enzyme as the reporter antibody (either directly or via a biotin-streptavidin complex) (Diamandis, 1991).

For two-site homosandwich assays, optimization of reagent concentrations, blocking non-specific binding sites, and proper washing of excess reagents are important. The significance of a proper blocking agent in the immunoassay design will be discussed later. Briefly, it was observed that the adequate blocking of non-specific sites by an effective blocking agent and multiple washings provide the additional low backgrounds and generated consistent signal stability (Figure 29).

The consistency in signal stability was further enhanced by multivalent interaction with the solid phase anti-LPS O157 antibody and identical repeating polysaccharide units of LPS O157 antigen. It is estimated that there are 2 million repeating LPS epitopes on a single *E. coli* cell. Thus the carbohydrates clustered together are held tightly to the solid phase. This would result in increased avidity resulting in signal stability.

In the majority of homosandwich immunoassays in use today, this signal is generated by a non-isotopic label conjugated to the reporter antibody (Gosling, 1990). Horseradish peroxidase conjugated to streptavidin is one of the most widely used enzymatic labels in immunoassays (Diamandis, and Christopoulos, 1991). In the presence of hydrogen peroxide, the enzyme acts by the chemical conversion of a substrate to a colorimetric, fluorometric, or luminescent signal (Kricka, 1988). The colorless tetramethylbenzidine (TMB) substrate reacts with $H_2O_2/HRPO_{catalyst}$ to generate a colorimetric product. It was estimated that 0.4 μg/well biotinylated reporter antibody gave sufficient colorimetric response of TMB substrate in evaluation of LPS analyte (Figure 32).

Fluorescence enzyme two-site sandwich immunoassay

The principle of fluorescence is in utilizing a fluorophore to absorb light at one wavelength and emit light at a longer wavelength. The emitted light is always at a longer wavelength than the absorbed light due to limited energy loss by the molecule in the excited electronic state (Hovius *et al.*, 2000).

To eliminate the influence of well-to-well light interference, the assay was carried out in a black 96 well microplate. The other approach in minimizing the background interference was by optimizing the concentration of LPS O157 antigen and tracer biotinylated P124 antibody. In this study, fluorogenic QuantaBlu™ substrate was used for the detection of horseradish peroxidase activity. The addition of QuantaBlu™ substrate to a streptavidin-peroxidase conjugated homosandwich assay resulted in the generation of a blue fluorescent product. Light absorption was optimal at 546 nm with emission at 575 nm. The data presented indicates that 0.4 μg/well tracer antibody was sufficient to achieve ample intensity of fluorophore signal (Figure 33).

Chemiluminescence enzyme two-site sandwich immunoassay

Chemiluminescence is the generation of visible light by a chemical reaction and as such does not use any light source. Thus the need for a complicated and inefficient optical wavelength filtering system is eliminated. Luminol reacts in the presence of hydrogen peroxidase as an oxidant and horseradish peroxidase as a catalyst. It is the most successful among enzyme chemiluminescent assays (Whitehead *et al.*, 1983). Most enzyme-mediated luminescent reactions produce weak and rapidly decaying emission (Kricka, 1988). Hence, new commercial substrate combinations such as Picotag™ and Femtotag™ have been developed for long lasting signal.

Background luminescence is an important characteristic in immunoassay sensitivity (Selby, 1999). To provide the low background and generate consistent signal stability, multicycle washing was applied. In addition to multicycle washing and blocking non-specific binding, the optimal concentration of reagent was also evaluated (Figure 34, and Figure 35). Using the concentration range from 4 –0.004 µg/well of tracer antibody bound to various amount of LPS O157 indicated that 0.4 µg/well was sufficient. The activity of peroxidase with Pico™ and Femto™ chemiluminescent substrate in the ELISA system was completed within 5 minutes of initiation the chemiluminescent reaction.

Colorimetric, fluorometric, and chemiluminescent signal substrate characteristics play an important role in the lower limits of the analyte or the amount of tracer monoclonal antibody needed for the testing. Figure 36 depicts the significant differences in terms of sensitivity between the different detection systems in an ELISA homosandwich system. Fluorescent QuantaBlu™ detection will allow more sensitive detection than colorimetric TMB substrate, but chemiluminesce Femto™ has been shown to be the most sensitive assay. The limited studies on sensitivities of LPS detection (Table 9) should be further validated in the future to develop a prototype diagnostic assay.

Blocking agents for ELISA

The choice of blocking agent can be vital to the level of sensitivity and specificity of immunoassay systems. Blocking the unreactive sites on the polystyrene surface after coating can minimize further non-specific binding, decreasing background and increasing sensitivity. A variety of agents have been used for blocking such as albumin, dry milk, casein, gelatins from pig and fish skin, lipoprotein, goat serum, thyroglobulin, and fetal calf serum (Vogt *et al.*, 1987). Studies have shown, that small molecules saturate the unoccupied binding sites better than larger molecules (Gibbs, 2001). The initial high background in ELISA system

($OD_{650} > 0.15$) suggested that trial and error is the only way in selecting the best possible candidate among the blocking agents for further experiments.

The results in Figure 29 demonstrate that different proteins have a different ability to block non-specific binding sites. Small molecules present in BSA could theoretically increase non-specific binding in immunosaays versus higher blocking potential of dialyzed bovine serum albumin (DBSA) where the undesired molecules are eliminated (Diamandis, 1996). Moreover, the addition of non-ionic detergent Tween 20 (0.01%) to DBSA (2%) blocking buffer further decreased background readings. The data presented demonstrates that utilizing a surfactant such as the nonionic detergent Tween 20 improved the blocking efficiency of DBSA to the level of the most efficient blocking agent which was the expensive FBS. The present study confirmed earlier reports of the blocking effect of Tween 20 in ELISA microwells (Steintz, 2000). It has been also observed that by leaving the antibody-coated microplates in blocking buffer for 4 days at 0 ^{0}C did not alter the binding ability of the antibody.

6. Conclusions and Future Work

1. The LPS O157 antigen was isolated and purified successfully by the modified method of Westphal and Jann for presented experiments. The biological activity of the LPS O157:H7, based on the standard curve of *E. coli* O113:H10 by *Limulus* amoebocyte lysate test, was comparable to those of internal control standard endotoxins of LPS O157 NRC standard and LPS O30.

2. The present work conducted a study to assess the dose-response relationships in mice to develop monoclonal antibodies. Based on the obsereved dose-response relationships, a long-term immunization protocol was developed to immunize mice with glycolipid antigen LPS O157:H7.

3. A monoclonal antibody, P124, secreting hybridoma was developed, cloned and recloned. Specificity to the O157 strains was determined compared to three other laboratory strains of *E. coli*. However, further screening with clinical isolates is essential.

4. *E. coli* O157:H7 positive samples were evaluated by epifluorescence microscopy by indirectly labelled P124 monoclonal antibody. The results show that the MAb P124 labelled with FITC can be used for immunofluorescence based water sample testing. Testing on actual field samples or in collaboration with the public health and water testing laboratories are future areas of work.

5. The anti-LPS O157 MAb P124 was used to estimate *E. coli* O157:H7 heat killed whole cells and extracted LPS O157:H7 in a homosandwich format by colorimetric, fluorometric, and chemiluminescence immunoassay. The MAb P124 can be used in a simple chemiluminescence immunoassay to detect *E. coli* O157:H7 bacteria and LPS O157. This could be a sensitive test to detect O157 antigens in food, fecal samples, and in blood.

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